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PROTEINS PARTICIPATING IN RESTORATION FROM CYTOPLASMIC MALE STERILITY TO FERTILITY AND GENES CODING THEREFOR

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(54) [Title of the Invention]

PROTEINS PARTICIPATING IN RESTORATION FROM CYTOPLASMIC MALE STERILITY TO FERTILITY AND GENES CODING THEREFOR

[Claims]

1. Proteins participating in restoration from cytoplasmic male sterility to fertility, wherein the proteins contain 14 or more of pentatricopeptide repeat (hereafter, abbreviated as PPR) motifs; the PPR motif group is divided into three or more blocks; each block has at least two or more PPR motifs, respectively; and the block at a carboxy terminal (C-terminal) has four PPR motifs.

2. The proteins according to claim 1, where the number of PPR motif is 14 to 16.

3. The proteins according to claim 1 or 2, wherein the PPR motif group is divided into three blocks, and the blocks have five, seven and four PPR motifs in respective order from the amino terminal (N-terminal) side, respectively.

4. The proteins according to claims 1 to 3, wherein the fourth amino acid existing in the second PPR motif existing from the amino terminal (N-terminal) side is other than serine, threonine and cysteine.

5. The proteins according to claim 4, wherein the fourth amino acid existing in the second PPR motif from the amino terminal (N-terminal) is any of asparagine, glutamine, asparagine acid, glutamic acid and histidine.

6. The proteins according to any of claims 1 to 5 further containing a signal peptide sequence for migrating to mitochondria at the amino terminal, or a sequence comprising -LysAspGluLeu- at the carboxy terminal.

7. Proteins participating in restoration from cytoplasmic male sterility to fertility causing an occurrence of a gel shift to the transcription after making contact with a transcription of a cytoplasmic male sterile gene.

8. Any of the following proteins:
(1) a protein having the 80th to 687th amino-acid sequences among the amino-acid sequences described in SEQ ID NO 3; or
(2) a protein having an amino-acid sequence where one to multiple amino acids are deleted, added and/or substituted in the 80th to 687th amino-acid sequences among the amino-acid sequences described in SEQ ID NO 3 and participating in restoration from cytoplasmic male sterility to fertility.

9. Any of the following proteins:
(1) a protein having amino-acid sequences described in SEQ ID NO 3; or
(2) a protein having an amino-acid sequence where one to multiple amino acids are deleted, added and/or substituted described in SEQ ID NO 3 and participating in restoration from cytoplasmic male sterility to fertility.

10. The proteins according to claim 1 to 9, wherein the cytoplasmic male sterile individual has a cytoplasmic male sterilie gene of kosena radish (Raphanus sativus cv. Kosena) and/or Ogura radish (Raphanus sativus L.) or homologue thereof.

11. DNA coding for the proteins according to any of claims 1 to 10.

12. Any of the following DNA:
(1) DNA having base sequences described in SEQ ID NO 2; or
(2) DNA having base sequences where one to multiple bases are deleted, added and/or substituted in the base sequences described in SEQ ID NO 2 and participating in restoration from cytoplasmic male sterility to fertility; or
(3) DNA hybridizing with base sequences described in SEQ ID NO 2 under stringent conditions and participating in restoration from cytoplasmic male sterility to fertility.

13. Any of the following DNA:
(1) DNA having the 3,754th to 8,553th base sequences among the base sequences described in SEQ ID NO 1; or
(2) DNA having the 3,754th to 8,553th base sequences where one to multiple bases are deleted, added and/or substituted among the base sequences described in SEQ ID NO 1 and participating in restoration from cytoplasmic male sterility to fertility; or
(3) DNA hybridizing with DNA having the 3,754th to 8,553th base sequences among the base sequences described in SEQ ID NO 1 under stringent conditions and participating in restoration from cytoplasmic male sterility to fertility.

14. Any of the following DNA:
(1) DNA having base sequences described in SEQ ID NO 1; or
(2) DNA having base sequences where one to multiple bases are deleted, added and/or substituted among the base sequences described in SEQ ID NO 1, and participating in restoration from cytoplasmic male sterility to fertility; or
(3) DNA hybridizing with DNA having base sequences described in SEQ ID NO 1 under stringent conditions and participating in restoration from cytoplasmic male sterility to fertility.

15. The DNA according to any one of claims 11 to 14, wherein the cytoplasmic male sterile individual has a cytoplasmic male sterilie gene of kosena radish (Raphanus sativus cv. Kosena) and/or Ogura radish (Raphanus sativus L.) or has homologue thereof.

16. A vector containing the DNA according any of claims 11 to 15.

17. A transformant having the DNA according to any of claims 11 to 15 or the vector according to claim 16.

18. The transformant according to claim 17 being a transformed plant.

19. A method for restoring from cytoplasmic male sterility to fertility using the DNA according to any of claims 11 to 15.

20. A transformant enabling the control of expression of a cytoplasmic male sterilie gene by partially or entirely introducing the DNA described in any of claims 11 to 15 to a cell having a cytoplasmic male sterilie gene and having the DNA according to any of claims 11 to 15.

21. A maintenance method for a cytoplasmic male sterile line by utilizing the transformant according to claim 20.

22. A method for detecting a gene participating in restoration from cytoplasmic male sterility by using 15 mer to 50 mer of oligonucleotide primer arbitrarily set from the DNA according to any of claims 11 to 15 or at least 15 mer or more of probe comprising all or part of the DNA according to claims 11 to 15, and by confirming that a quantity of base sequences to be

amplified by the primer or a quantity of base sequences detected by the probe is one gene or more per genome in a target biological sample.

DETAILED DESCRIPTION OF THE INVENTION

[0001]

[Industrial Field of Application]

The present invention relates to genes participating in restoration of cytoplasmic male sterility to fertility. In further detail, the present invention relates to genes participating in the restoration of cytoplasmic male sterility (hereafter, it may be abbreviated as 'cms') character to be utilized for the purpose of F1 hybrid (hereafter, referred to as F1) breed development; a vector containing said genes; and a transformant.

[0002]

[Prior Art]

In cereal crops, such as grain crops or vegetables, F1 breed development has caught on based upon the characteristics including 1) superior agronomic character due to hybrid vigor, 2) uniformity of harvest, and 3) protection of profits by breed cultivators because the genetic character is separated in the next generation, and this is practically used in many main crops.

[0003]

As one of methods for producing F1 variety seeds, a cms/Rf seed production system comprising the cytoplasmic male sterility (cms) line and a line for restoring the male sterility (hereafter, this may be abbreviated as 'Rf') is available, and for example, this system is developed with cereal crops, such as rice, sorghum or corn, and oil crops, such as sunflower; however, these are all developed using a technique, such as hybridization or cell fusion.

[0004]

In the meantime, F1 seed production system using self-incompatibility has been widely utilized in cruciferous; however, regarding rapeseed, because it has no stable self-incompatibility, another F1 seed production system utilizing the cms line and the Rf line is in demand.

[0005]

In the meantime, a study where the cytoplasmic male sterility (kosena-cms) derived from kosena radish (Raphanus sativus cv. Kosena) and the cytoplasmic male sterility (ogura-cms) derived from ogura radish (Raphanus sativus L.) are used in rapeseed has been continued in recent years. Both cms genes are coded to the genome of mitochondria, which are cytoplasmic organelle, and their base sequences are also known; however, for radish, since molecular-biological research is not in progress and markers that are required for gene isolation are hardly known, as well, it is difficult to isolate genes from the nuclear, and regarding Rf, it is merely introduced to rapeseed using techniques of hybridization or cell fusion from a fertility restoration system for radish.

[0006]

In addition, regarding the Rf gene, one or multiple restoring genes depending upon each cms system of plants. In the radish, it is necessary for the Rf1 and Rf2 genes simultaneously exist for the fertility restoration, and in addition, it is known that the Rf1 gene drastically decreases accumulation of ORF125 protein (M. Iwabuchi et al. Plant Mol. Biol. 39:183-188, 1999) within the mitochondria known as a cms causal protein of radish (Japanese Journal of Breeding 47 (supplementary volume 1) p. 186, 1997, Japanese Journal of Breeding 48 (supplementary volume 1) p. 197, 1998).

[0007]

Further, in the rapeseed, it is known that the Rf1 gene of radish introduced by hybridization or cell fusion according to a gene analysis test decreases the accumulation of ORF125 or ORF138 protein (M. Grelon et al. Mol. Gen. Genet. 243:540-547), which is known as a cms causal protein, and the decrease in the accumulation and the phenomenon of restoring the fertility in this ORF125 or ORF138 protein are completely matched (N. Koizuka, et al. Theor Appl Genet, 100:949-955, 2000). In other words, in order to restore the fertility with the rapeseed male sterile line, it is essential to decrease the accumulation of ORF125 or ORF138 protein, and the Rf1 gene is the important gene for this purpose.

However, for base sequence of the Rf gene, although only the Rf2 gene, which is one of restoring genes for T-cytoplasm that is one of corn cms, is identified and isolated; however, any base sequence of Rf genes in other plants is not known at all.

[0008]

[Problem to be Solved by the Invention]

In the rapeseed restoring line where Rf1 gene derived from the Ogura radish (Raphanus sativus L.) has been introduced by hybridization or cell fusion and the F1 variety that has been produced by said line as a father, the glucosinolate (hereafter, abbreviated as GSL) content becomes higher than a regulation value, and this is a practical problem. It appears that because the radish-derived gene participating in the GSL biosynthesis exists in the vicinity of the Rf1 gene and is genetically linked strongly, the GSL content of the rapeseed restoring line (Rf line) is increased. GSL is contained in oil expression, and since it is known that when GSL is supplied to animals as feed, it causes enlarged thyroid gland, the GSL content of rapeseed is

required to be 18 μmole/g in North America and 20 μmole/g or less in Europe during development of new varieties plants.

[0009]

In addition, in recent years, the development of plants where a function, such as herbicide resistance, is added due to genetic recombination is on the move, and in order to efficiently create these plants, only the presence of rapeseed restoration line obtained by hybridization or cell fusion is not sufficient, and the isolation of Rf gene, in particular, radish-derived Rf1 gene has been in demand.

[0010]

In other words, isolation of the Rf gene, in particular, the radish-derived Rf1 gene, and identification of the structure thereof were designated as a problem to be solved. In addition, another problem to be solved in the present invention is to provide a means for establishing the rapeseed restoration line by utilizing the isolated Rf gene.

[0011]

[Means for Solving the Problem]

As a result of a keen examination for solving the problems, the inventors of the present invention have succeeded to clone the Rf1 genes from radish and rapeseed, and to solve the problems.

In other words, according to the present invention, proteins participating in restoration from cytoplasmic male sterility to fertility, wherein the proteins contain 14 or more of pentatricopeptide repeat (hereafter, abbreviated as PPR) motifs; the PPR motif group is divided into three or more blocks; each block has at least two or more PPR motifs,

respectively; and the block at a carboxy terminal (C-terminal) has four PPR motifs are provided.

[0012]

According to preferred embodiments of the proteins,

proteins whose number of PPR motifs is 14 to 16;

proteins where the PPR motif group is divided into three blocks, and the blocks have five, seven and four PPR motifs in respective order from the amino terminal (N-terminal) side, respectively;

proteins where the fourth amino acid existing in the second PPR motif existing from the amino terminal (N-terminal) side is other than serine, threonine and cysteine;

proteins where the fourth amino acid existing in the second PPR motif from the amino terminal (N-terminal) is any of asparagine, glutamine, asparagine acid, glutamic acid and histidine; and

proteins further containing a signal peptide sequence for migrating to mitochondria at the amino terminal, or a sequence comprising -LysAspGluLeu- at the carboxy terminal;

are provided.

[0013]

According to another aspect of the present invention, proteins participating in restoration from cytoplasmic male sterility to fertility causing an occurrence of a gel shift to the transcription after making contact with a transcription of a cytoplasmic male sterile gene.

According to another aspect of the present invention, either of the following proteins is provided:

(1) a protein having the 80th to 687th amino-acid sequences among the amino-acid sequences described in SEQ ID NO 3; or

(2) a protein having amino-acid sequences where one to multiple amino acids are deleted, added and/or substituted in the 80th to 687th amino-acid sequences among the amino-acid sequences described in SEQ ID NO 3 and participating in restoration from cytoplasmic male sterility to fertility.

[0014]

According to another aspect of the present invention, either of the following proteins is provided:

(1) a protein having amino-acid sequences described in SEQ ID NO 3; or

(2) a protein having an amino-acid sequence where one to multiple amino acids are deleted, added and/or substituted described in SEQ ID NO 3 and participating in restoration from cytoplasmic male sterility to fertility.

In the present invention, preferably, the cytoplasmic male sterile individual has a cytoplasmic male sterilie gene of kosena radish (Raphanus sativus cv. Kosena) and/or Ogura radish (Raphanus sativus L.) or homologue thereof.

[0015]

According to another aspect of the present invention, DNA coding for the proteins of the present invention is provided.

According to another aspect of the present invention, any of the following DNA is provided:

(1) DNA having base sequences described in SEQ ID NO 2; or

(2) DNA having base sequences where one to multiple bases are deleted, added and/or substituted in the base sequences described in SEQ ID NO 2 and participating in restoration from cytoplasmic male sterility to fertility; or

(3) DNA hybridizing with base sequences described in SEQ ID NO 2 under stringent conditions and participating in restoration from cytoplasmic male sterility to fertility.

[0016]

According to another aspect of the present invention, any of the following DNA is provided:

(1) DNA having the 3,754th to 8,553th base sequences among the base sequences described in SEQ ID NO 1; or

(2) DNA having the 3,754th to 8,553th base sequences where one to multiple bases are deleted, added and/or substituted among the base sequences described in SEQ ID NO 1 and participating in restoration from cytoplasmic male sterility to fertility; or

(3) DNA hybridizing with DNA having the 3,754th to 8,553th base sequences among the base sequences described in SEQ ID NO 1 under stringent conditions and participating in restoration from cytoplasmic male sterility to fertility.

[0017]

According to another aspect of the present invention, any of the following DNA is provided:

(1) DNA having base sequences described in SEQ ID NO 1; or

(2) DNA having base sequences where one to multiple bases are deleted, added and/or substituted among the base sequences described in SEQ ID NO 1, and participating in restoration from cytoplasmic male sterility to fertility; or

(3) DNA hybridizing with DNA having base sequences described in SEQ ID NO 1 under stringent conditions and participating in restoration from cytoplasmic male sterility to fertility.

According to another aspect of the present invention, the cytoplasmic male sterile individual has a cytoplasmic male sterilie gene of kosena radish (Raphanus sativus cv. Kosena) and/or Ogura radish (Raphanus sativus L.) or has homologue thereof.

[0018]

According to another aspect of the present invention, a vector containing the DNA of the present invention is provided.

According to another aspect of the present invention, a transformant having the DNA of the present invention or the vector of the present invention is provided. The transformant is preferably a transformed plant.

According to another aspect of the present invention, a method for restoring from cytoplasmic male sterility to fertility characterized by using the DNA of the present invention is provided.

According to another aspect of the present invention, a transformant enabling the control of expression of a cytoplasmic male sterilie gene by partially or entirely introducing the DNA of the present invention to a cell having a cytoplasmic male sterilie gene and having the DNA of the present invention is provided.

According to another aspect of the present invention, a maintenance method for a cytoplasmic male sterile line by utilizing the transformant is provided.

[0019]

According to another aspect of the present invention, a method for detecting a gene participating in restoration from cytoplasmic male sterility by using 15 mer to 50 mer of oligonucleotide primer arbitrarily set from the DNA of the present invention or at least 15 mer or more of probe comprising all or part of the DNA of the present invention, and by confirming that a quantity of base sequences to be amplified by the primer or a quantity of base sequences detected by the probe is one gene or more per genome in a target biological sample is provided.

[0020]

[Embodiment of the Invention]

Hereafter, embodiments of the present invention will be described in detail.

(1) Mode of protein of the present invention

The protein of the present invention relates to any proteins from (i) to (iv).

(i) Proteins participating in restoration from cytoplasmic male sterility to fertility, wherein the proteins contain 14 or more of pentatricopeptide repeat (hereafter, abbreviated as PPR) motifs; the PPR motif group is divided into three or more blocks; each block has at least two or more PPR motifs, respectively; and the block at a carboxy terminal (C-terminal) has four PPR motifs;

(ii) Proteins participating in restoration from cytoplasmic male sterility to fertility causing an occurrence of a gel shift to the transcription after making contact with a transcription of a cytoplasmic male sterile gene.

(iii) Any of the following proteins:

(1) A protein having the 80th to 687th amino-acid sequences among the amino-acid sequences described in SEQ ID NO 3; or

(2) A protein having an amino-acid sequence where one to multiple amino acids are deleted, added and/or substituted in the 80th to 687th amino-acid sequences among the amino-acid sequences described in SEQ ID NO 3 and participating in restoration from cytoplasmic male sterility to fertility; and

(iv) Any of the following proteins:

(1) A protein having amino-acid sequences described in SEQ ID NO 3; or

(2) A protein having an amino-acid sequence where one to multiple amino acids are deleted, added and/or substituted described in SEQ ID NO 3 and participating in restoration from cytoplasmic male sterility to fertility.

[0021]

In this specification, the PPR motif represents "Pentatricopeptide Repeat" motif. This PPR motif is a motif structure of a new protein discovered as a result of the progress of the genome project of Arabidopsis. The basic motif is such that 35 degenerated amino acid sequences are tandem-repeated on the primary structure of the protein. The PPR motif has a sequence represented by an amino terminal (N-terminal)–"VTYNTLISGYCKNGKLEEALELFEEMKEKGIKPDV"–carboxy terminal (C-terminal) as a consensus amino acid sequence. This motif is proposed by Small and Peeters (reference: Trends Biochem Sci 2000, 25 46-47), and approximately 200 genes that can adopt this motif in the genome of Arabidopsis are reported in the gene bank, such as GenBank (http//www.ncbi.nlm.nih.gov/Genbank/index.html) at that time when this reference was published. At present, a determination about whether or not one arbitrary protein can have this motif structure can be easily determined by a program in Protein families' database of alignments and HMNs (hereafter, abbreviated as Pfam, http//:www.sanger.ac.uk/Software/Pfam/search.shtml) located in Sanger Institute, UK.

[0022]

Examples where a function of the protein having the PPR motif has become clear up to the present date include 1) an example where fermentum PET309, which is a protein to transfer to mitochondria, or Neurospora crassa CYA-5 interacts with cox1 mRNA, which is a mitochondrion gene, and the expression of cox1 is controlled by processing after transcription or in a translation level (Manthey and McEwen EMBO J 1995 14 4031-4043, Coffin et.al. Curr Genet1997 32 273-280) and 2) another example where corn CRP1, which is a PPR motif protein to transfer to chloroplast, is essential for translation of petA and petD genes, which are chloroplast genes, and in

addition, is essential for a step of petDmRNA processing, as well (Fisk et. al. EMBO J 1999 18 2621-2630); therefore, it is assumed that it is highly possible that the protein having the PPR motif is involved in the translation control in some form.

[0023]

In this occasion, the inventors of the present application isolated the genes participating in the restoration from the sterility of kosena radish (Raphanus sativus cv. Kosena) cytoplasmic male sterile individual to fertility, and they discovered that the proteins coded by the genes have 14 or more of pentatricopeptide repeat (hereafter, abbreviated as PPR), and the PPR motif group is divided into three or more blocks, and in addition, each block has at least two or more PPR motifs, and, the block existing at the closest to the carboxy terminal (C-terminal) has four PPR motifs.

[0024]

As the protein participating in the restoration from the sterility of cytoplasmic male sterile individual to fertility, a protein whose number of PPR motifs is 14 to 16 is preferable, and a protein where the PPR motif group is divided into three blocks and each block has 5, 7 and 4 PPR motifs in respective order form the amino terminal (N-terminal) side is more preferable.

Specifically, proteins comprises

(1) PPR cluster #1: a PPR cluster comprising 175 residues where the first PPR motif to the fifth PPR motif from the N-terminal are continuous,

(2) PPR cluster #2: a PPR cluster comprising 245 residues where the sixth PPR motif to the twelfth PPR motif from the N-terminal are continuous, and

(3) PPR cluster #3: a PPR cluster comprising 140 residues where the thirteenth PPR motif to the sixteenth PPR motif from the N-terminal are continuous.

[0025]

The protein where the fourth amino acid existing in the second PPR motif from the amino terminal (N-terminal) is other than serine, threonine or cysteine is further preferable, and the protein where the fourth amino acid existing in the second PPR motif from the amino terminal (N-terminal) side is any of asparagine, glutamine, asparagine acid and histidine is further preferable, and the protein where the fourth amino acid existing in the second PPR motif from the amino terminal (N-terminal) is asparagine is the most preferable.

[0026]

Since it is normally known that the fertility gene exists in a nuclear genome and the cytoplasmic male sterility exists in mitochondria, it is preferable that the protein participating in the restoration from sterility of the cytoplasmic male sterile individual to fertility has a signal peptide sequence at the amino terminal for transferring to the mitochondria or has a sequence comprising 'LysAspGluLeu' at the carboxy terminal.

[0027]

The signal peptide at the N-terminal for transferring to the mitochondria includes the one to be confirmed by a prediction program "TargetP" (http://www.cbs.dtu.dk/services/TargetP/) based upon the algorism of O. Emanuelsson et al. (J. Mol. Biol. 300, 1005-1016 (2000)) or the one to be confirmed by another prediction program "Psort" (HYPERLINK http://psort.nibb.ac.jp) http://psort.nibb.ac.jp/, and a specific example of the signal peptide includes a signal peptide of Arabidopsis AtOXA1 (W. Sakamoto et al.: Plant Cell Physiol, 41:1157-1163) (MetAlaPheArgGlnThrLeuSerIleArgSerArgLeuPheAlaArgArgAsnGlnProVal TyrHisIleIleProArgGluSerAspHisGluArgAsp). Among them, 1 to 79 of amino acid sequences among the amino sequences described in SEQ ID NO 3 are

preferable, and 1 to 34 of amino acid sequences among the amino sequences described in SEQ ID NO 3 are especially preferable.

[0028]

Further, the proteins participating in the restoration from sterility of cytoplasmic male sterile individual to fertility induces a translation inhibition of the cytoplasmic male sterilie gene by combining with the transcription of the cytoplasmic male sterilie gene, and restores the sterility of the cytoplasmic male sterile individual to fertility.

[0029]

The transcription of the cytoplasmic male sterilie gene includes transcription (mRNA) of genes of ORF125, which is a causal protein causing the kosena cytoplasmic male sterility, or ORF138, which is a causal protein causing the ogura cytoplasmic male sterility, and 5'-UTR (Bonhomme et al; Mol Gen Genet, 235:340-348(1992)) region of the gene is preferable.

A method to confirm about whether or not combining with the transcription of cytoplasmic male sterilie gene includes a method where the protein of the present invention is added to mRNA of ORF125 or ORF 138, which was artificially transcripted in vitro, and the mixture is electrophoresed, and a so-called gel shift method, and as specific operation procedures, the gel shift method may be conducted under normal conditions.

[0030]

Further, another method to confirm whether or not the expression is restrained by adding the protein of the present application to the expression where a fused gene between ORF125 or ORF138 gene and a detectable reporter gene, such as Beta-galactosidase or luciferase, is expressed in Escherichia coli is also included.

Specifically, incorporation of a fertility gene shown with the base sequence of SEQ ID NO 2 into Escherichia coli expression vector and another incorporation of a vector where a 5'-UTR region of ORF125 and coding part of 25 amino acids are fused to the LacZ gene into the Escherichia coli are prepared, and if the expression is induced, the expression of the LacZ gene is restrained only when the expression vector where the fertility gene is incorporated coexists, and the blue Escherichia coli colony becomes white in the presence of X-Gal. As described above, the existence of the function to restore the sterility of the cytoplasmic male sterility to fertility by inducing the translation inhibition of the cytoplasmic male sterilie gene can be confirmed via the confirmation by utilizing the gene that codes the protein of the present application.

[0031]

The most preferable protein among proteins of the present invention participating in the restoration from sterility of cytoplasmic male sterile individual to fertility includes
(1) a protein having the 80th to 687th amino acid sequences among the amino acid sequences described in SEQ ID NO 3; or (2) a protein having amino acid sequences where one to multiple amino acids are deleted, added and/or substituted in the 80th to 687th amino acid sequences among the amino acid sequences described in SEQ ID NO 3 and participating in the restoration from sterility of cytoplasmic male sterile individual to fertility, and the proteins having transfer sequences to mitochondria in the sequences include (1) a protein having the amino acid sequences described in SEQ ID NO 3; or (2) a protein having amino acid sequences where one to multiple amino acids are deleted, added and/or substituted in the amino acid sequences described in SEQ ID NO 3 and participating in the restoration from sterility of cytoplasmic male sterile individual to fertility.

[0032]

The protein of the present invention is a protein that can participate in the restoration from sterility of cytoplasmic male sterile individual to fertility. More specifically, when a transformed plant (Rf line) where DNA that codes for the protein of the present invention is bred with an individual of the cytoplasmic male sterile line (cms line), F1 seeds where the fertility has been restored can be obtained. As preferable individuals of the cms line, individuals where the cytoplasmic male sterilie gene(s) of kosena radish (Raphanus sativus cv. Kosena) and/or Ogura radish (Raphanus sativus L.) is introduced are included.

The protein of the present invention can be screened and isolated by utilizing the gel shift method or can be isolated or synthesized by utilizing DNA of the present invention to be described below. The acquisition method for the protein of the present invention will be described later.

[0033]

(2) DNA mode of the present invention:

The DNA of the present invention relates to any of DNA from the following (i) to (iv):

(i) DNA coding for the proteins of the present invention;

(ii) any of the following DNA:

(1) DNA having base sequences described in SEQ ID NO 2; or

(2) DNA having base sequences where one to multiple bases are deleted, added or substituted in the base sequence described in SEQ ID NO 2 and participating in the restoration from sterility of an cytoplasmic male sterility to fertility; or

(3) DNA hybridizing with DNA having base sequences described in SEQ ID NO 2, and participating in the restoration from sterility of a cytoplasmic male sterile individual to fertility,

(iii) any of the following DNA:

(1) DNA having the 3,754th to 8,553rd base sequences, in base sequences described in SEQ ID NO 1; or

(2) DNA having base sequences where one to multiple bases are deleted, added or substituted the 3,754th to 8,553rd base sequences, in base sequences described in SEQ ID NO 1; or

(3) DNA hybridizing base sequences having the 3,754th to 8,553rd base sequences described in SEQ ID NO 1 under stringent conditions, and participating in the restoration from sterility of an cytoplasmic male sterile individual to fertility, or

(iv) any of the following DNA:

(1) DNA base sequences described in SEQ ID NO 1; or

(2) DNA having base sequences where one to multiple bases are deleted, added or substituted in base sequences described in SEQ ID NO 1; or

(3) DNA hybridizing base sequences described in SEQ ID NO 1 under stringent conditions, and participating in the restoration from sterility of a cytoplasmic male sterile individual to fertility.

[0034]

In this specification, the DNA of the present invention may be referred to as a gene of the present invention.

The base sequences shown with SEQ ID NO 1 are genome DNA base sequences comprising 8,553 bases; and base sequences shown in SEQ ID NO 2 are code sequences acquired from SEQ ID NO 1. SEQ ID NO 3 includes amino acid sequences coded by the base sequences shown with SEQ ID NO 2.

[0035]

In this specification, "base sequences where one to multiple base sequences are deleted, added and/or substituted" mean, for example, that any of 1 to 20 base sequences, preferably 1 to 15 base sequences, more preferably

1 to 10 base sequences, further preferably 1 to 5 base sequences are deleted, added and/or substituted.

In this specification, "amino acid sequences where one to multiple base sequences are deleted, added and/or substituted" mean, for example, that any of 1 to 20 amino acids, preferably 1 to 15 amino acids, more preferably 1 to 10 amino acids, further preferably 1 to 5 amino acids are deleted, added and/or substituted.

[0036]

In this specification, "DNA hybridizing under stringent conditions" means a base sequence of DNA acquired by a colony hybridization method, a plaque hybridization method or a Southern blot hybridization method using DNA as a probe, and for example, after hybridization is conducted at 65 degrees C in presence of 0.7 to 1.0 M NaCl using a filter where a colony-derived or plaque-derived DNA or fragment of DNA is immobilized, DNA that can be identified by cleaning a filter under a condition at 65 degrees C using 0.1 to 2 × SSC solution (composition of 1 × SSC is 150 mM sodium chloride and 15 mM sodium citrate).

[0037]

The hybridization can be conducted based upon a method described in Molecular Cloning: A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989 (hereafter, abbreviated as "Molecular Cloning 2nd Ed.).

[0038]

DNA hybridizing under stringent conditions includes DNA having a certain level or more homology with the base sequence of DNA used as a probe. The 'certain level or more homology' herein is for example, 70 % or more, preferably 80 % or more, more preferably 90 % or more, further

preferably 93 % or more and the particularly preferably 95 % or more and the most preferably 97 % or more. Furthermore, the DNA having a certain level or more of homology herein contains both polynucleotide having the above-mentioned homology and complementary strand polynucleotide thereof.

[0039]

DNA of the present invention is DNA that can participate in the restoration from sterility of cytoplasmic male sterile individual to fertility. More specifically, F1 seeds where fertility is restored can be obtained by breeding a transformed plant (Rf line) where DNA of the present invention is introduced with individuals of cytoplasmic male sterile line (cms line). The individuals of the cms line preferably include an individual where a cytoplasmic male sterile gene of kosena radish (Raphanus sativus cv. Kosena) and/or Ogura radish (Raphanus sativus L.) is introduced.

[0040]

(3) Acquisition method for DNA of the present invention:

The acquisition method for DNA of the present invention is not particularly limited. The DNA of the present invention can be isolated or synthesized by utilizing a general breeding technique and a general genetic technique, which are well-known to a person with ordinary skills in the art pertaining to the present invention, based upon information of amino acid sequences to be obtained by combining PPR motif and mitochondrion transfer sequence obtained based upon amino acid sequences described in SEQ ID NO 1 or SEQ ID NO 2 disclosed in this specification and amino acid sequences described in SEQ ID NO 3 or information of amino acid sequences described in SEQ ID NO 3.

[0041]

Specifically by appropriate plant origin where the genes of the present invention are expressed, specifically by genus Raphanus plants including radish variety and related species thereof or other plants where the genome DNA including a cytoplasmic male sterile restoring gene has been transferred from these plants by techniques of hybridization or cell fusion; more specifically by (Raphanus sativus cv. Kosena), Ogura radish (Raphanus sativus L.) and Yuanhong radish (Raphanus sativus cv. Yuanhong) or genus Raphanus plants, such as these radish varieties and related species or techniques of hybridization or cell fusion, the genome DNA including a cytoplasmic male sterility restoring gene of these plants can be obtained, and for example, genes of the present invention can be acquired by isolating a DNA marker positioned in the vicinity of the Rf gene, by preparing a genome map showing the relationship of genetic distance between this DNA marker and the Rf gene, and by a positional cloning method (also referred to as chromosome walking) of the Rf region as a starting point of the genome map.

[0042]

This method is started from discovering appropriate DNA marker on the genome DNA, and producing a genome map by measuring a genetic distance between the Rf gene and the DNA marker. It is necessary for the DNA marker to identify between the father-derived genome and the mother-derived genome, and it generally comprises the length of number 100 bp. Further, it is necessary for the DNA marker to place on the same chromosome, and a marker whose hereditary mode is substantially the same because the distance in between the gene is close, i.e., a marker that is genetically strongly linked is desirable.

[0043]

The RFLP method has been conventionally used as the DNA marker isolation method; however, the RAPD method and AFLP (amplified fragment length polymorphism) method (Nucleic Acids Research, 1995, Vol. 23, No. 21, 4407-4414), which are simplified methods using PCR, are used. In particular, the AFLP method is effective as a means to obtain a marker that is genetically strongly linked. As a material to measure a genetic distance with a marker, an F2 aggregate to be obtained by self-pollinating the F1 generation where a recessive homo-individual not having a Rf1 gene and a dominant homo-individual having the Rf1 gene in the homo-individual and a BC1 aggregate to be obtained by breeding the F1 generation and the recessive homo-individual, which is a parent of the F1 generation and does not have a target gene can be normally used.

[0044]

As the recessive homo-individual, radish variety of cytoplasmic male sterile line, genus Raphanus plants including related species, to be more specific, kosena radish (Raphanus sativus cv. Kosena) and Ogura radish (Raphanus sativus L.) in the cytoplasmic male sterile line; or genus Brassica plants where kosena radish (Raphanus sativus cv. Kosena)-derived cytoplasmic male sterility (kosena cms) or Ogura radish (Raphanus sativus L.)-derived cytoplasmic male sterility (Ogura cms) is transferred, to be more specific, cms rapeseed can be used.

[0045]

As the dominant homo-individual, radish variety, which is the Rf line, genus Raphanus plants including related species, to be more specific, kosena radish (Raphanus sativus cv. Kosena), Ogura radish (Raphanus sativus L.) and Raphanus sativus; or genus Brassica plants where genome DNA including a cytoplasmic male sterility restoring gene of genus Raphanus

plants including these radish varieties and related species is transferred by a technique of hybridization or cell fusion, to be more specific, Rf rapeseed can be used.

[0046]

For the F2 aggregate to be obtained by self-pollinating the F1 generation, which is obtained by breeding these parents, and the BC1 aggregate to be obtained by breeding the F1 generation and the recessive homo-individual, it is desirable to analyze 100 individuals normally and to analyze 1,000 individuals more preferably, and the more the number of individuals is increased, the greater the accuracy of the genome map becomes, and the physical distance from the DNA marker to the target gene becomes shorter. As similar to the case of the Rf gene, it becomes possible to obtain a DNA marker with shorter physical distance.

[0047]

As a material for measuring the genetic distance between the DNA marker and the Rf gene, for example, thousands of F2 aggregates obtained by self-pollinating the radish F1 generation where cms line kosena radish (Raphanus sativus cv. Kosena) and Rf line Yuanhong radish (Raphanus sativus cv. Yuanhong) were bred based upon a method described in N. Koizuka, et al. Theor Appl Genet, 100:949-955, 2000 can be used. The DNA markers that are linked at positions with approximately 0.2 cM of genetic distance at both sides in the form of interposition of the Rf gene can be isolated by analyzing these, and a genome map showing the genetic distance between the marker and the Rf gene as shown in Fig. 1 can be prepared.

[0048]

Following the preparation of the genome map, the genome DNAs corresponding to the positions are cloned, and it becomes possible to connect

the DNA markers by sandwiching the target gene. Normally, since the physical distance between the DNA marker and the target gene is great, the target gene region from the DNA marker shall be covered by connecting multiple clones having genome DNA fragments. The process to connect between these DNA markers with the clones is a contig production. Similar to the case of the Rf gene, the contig can be produced by connecting multiple clones having the genome DNA fragments so as to cover the Rf gene region in between the DNA markers existing close to the Rf gene.

[0049]

An aggregate of the clones having the genome DNA fragments can be obtained by producing a genomic library. Normally, several types of vectors are used according to the length of clonable genome DNA, and for example, a library utilizing a lambda pharge vector that can clone fragments up to approximately 20 kb, a cosmid vector that can clone comparatively longer fragments (up to 40 kb), and a BAC (bacterial artificial chromosome) that can clone longer fragments, such as, 100 kb or longer of fragments can be mentioned.

[0050]

In any library, it is important that a value where the average length of the cloned fragments is multiplied by the number of aggregates in the library becomes a value, which is four or five times of the entire length (genome size) of genome supplied to the library. Since it is believed that the genome size of radish is approximately 500 Mbp, in the case that the average length is 20 kb as in the lambda pharge vector, the number of aggregates becomes 1.0×10^5 to 1.25×10^5, and in the case that the average length is 40 kb in the cosmid library, the number of aggregates becomes 5.0×10^4 to 6.25×10^4. Since it is believed that the genome size of rapeseed is approximately 1,000 Mbp, in the case that the average length is 20 kb in the lambda pharge vector, the

number of aggregates becomes 2.0×10^5 to 2.5×10^5, and in the case that the average length is 40 kb in the cosmid library, the number of aggregates becomes 1.0×10^5 to 1.25×10^5.

[0051]

The genome DNA to be provided to the library may be extracted from a living organism containing the target gene using a conventional method. In the case of Rf gene, the radish variety, which is a Rf line, genus Raphanus plants including related species, to be more specific, kosena radish (Raphanus sativus cv. Kosena), Ogura radish (Raphanus sativus L.) and Yuanhong radish (Raphanus sativus cv. Yuanhong), which are cytoplasmic male sterility restoring lines; or genus Brassica plants where the genome DNA containing a cytoplasmic male sterility restoring gene of genus Raphanus plants including these radish species and related species is transferred by a technique of hybridization or cell fusion, and to be more specific Rf rapeseed can be utilized. In general, it is believed that it is the most desirable that the genome DNA is extracted from a plant, which is the same Rf line as the parent that was utilized at the time of producing the F2 aggregate or the BC1 aggregate, and a genomic library is produced. The genome DNA can be prepared by following a conventional method, such as CTAB method (Murray, M. G. and Thompson, W. F. (1980) NucleicAcids Res., 8, 4321).

[0052]

In the contig production, at first, a clone maintaining DNA markers located at both sides of the Rf gene is isolated. The clone is isolated from the genomic according to a conventional method, using a plaque hybridization method in the case of the lambda pharge library or using a colony hybridization method in the case of the cosmid library and the BAC library. Next, a contig is produced by isolating clones adjacent to the clone using the

terminal region of the isolated clone as an index. After the production, the base sequence of the contig is determined according to a conventional method.

[0053]

Due to the recent progress of the genome project, a technology to estimate functioning genes from base sequences of the genome DNA has been developing. The gene discovery program represented by "Genscan" can estimate genes with considerably high accuracy. Further, the homology search program represented by "BLAST" can estimate similarity of other genes and proteins. Utilizing this analysis software, target genes are estimated and isolated. Even in the case of the Rf gene, it is possible to similarly isolate and identify genome DNA sequences of contig by utilizing similar analysis software. Further, analyzing them, a promoter part on the genome DNA base sequence, a structural gene part including intron, and a terminator part are expressed. Further, simultaneously, genes to be translated into a protein except for intron and amino acid sequences to the gene are expressed. As described above, it is possible to estimate the Rf genes on the contig with considerably high accuracy.

[0054]

Further, in order to confirm whether or not what form the target genome is actually expressed within a living organism based upon the estimated gene sequence by utilizing the analysis software, mRNA is purified and it can be proved by isolating a supplementary DNA (cDNA) to this mRNA. Further, regarding from where the transcription has started, it is possible to prove by analyzing with a method where PCR is applied referred to as 5'-RACE method as a simplified method, and to be more certain, with the primer extension method or the S1 mapping method.

The above-mentioned methods are described in Molecular Cloning: A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

[0055]

The isolated gene of the present invention based upon the base sequences estimated by the techniques specifically includes DNA shown in SEQ ID NO 2, and it also becomes possible to easily isolate cDNA from other plant origins by a general genetic technical based upon the DNA sequence.

[0056]

Specifically by appropriate plant origin where the genes of the present invention are expressed, specifically by Genus Raphanus plants including radish variety and related species thereof or other plants where the genome DNA including a cytoplasmic male sterile restoring gene has been transferred from these plants by techniques of hybridization or cell fusion; more specifically by (Raphanus sativus cv. Kosena), Ogura radish (Raphanus sativus L.) and Yuanhong radish (Raphanus sativus cv. Yuanhong) or Genus Raphanus plants, such as these radish varieties and related species or techniques of hybridization or cell fusion, a cDNA library is prepared in accordance with a usual method from the genus Brassica plants where the genome DNA including a cytoplasmic male sterility restoring gene of these plants, and cDNA equivalent to the gene of the present invention can be isolated using appropriate DNA fragments, which are unique to the gene of the present invention, as a probe, or by selecting the desired clones using an antibody to the translation product of the gene of the present invention.

[0057]

In the above-mentioned description, as an origin of cDNA, various cells, tissues and derived cultured cells where the gene of the present invention is

expressed are illustrated. Further, separation of entire RNA from these, separation and purification of mRNA, acquisition of cDNA and cloning can be all conducted in accordance with a conventional method.

The method of screening the gene of the present invention from the cDNA library is also not limited, but a normal method can be followed.

[0058]

As the probe used herein, DNA, which is chemically synthesized based upon the information regarding the base sequences of the gene of the present invention, can be generally used; however, the gene of the present invention, which has already been acquired, and fragments thereof can be excellently used. Further, a sense primer and an anti-sense primer, which are set based upon the base sequence information of the gene of the present invention, can also be used as a probe for screening.

[0059]

The nucleotide sequences of the sense primer and anti-sense primer used for the primer include partial nucleotide sequences corresponding to the DNA coding for the amino acid sequences shown in SEQ ID NO 3, and the one having at least 15 to 50 successive bases, preferably 20 to 30 successive bases can be mentioned. Alternatively, a positive clone itself having the sequences can be used as a probe.

[0060]

On the occasion of acquiring the gene of the present invention, a technique, which is normally used for the isolation of genes, may be used by combining with a DNA/RNA amplification method according to the PCR method or the RACE method represented by the 5'-RACE method.

[0061]

The primer used on the occasion of adopting the PCR method can be appropriately set based upon the sequence information of the gene of the present invention, which has been clarified by the present invention, and this can be synthesized in accordance with a conventional method. Furthermore, for isolation and purification of amplified DNA/RNA fragments, a conventional method can be used as described above, and for example, it can be conducted with gel electrophoresis.

[0062]

The gene of the present invention obtained as described above or various DNA fragments can determine their base sequences in accordance with a conventional method.

According to the gene of the present invention obtained as described above, a presence of the gene of the present invention in an individual or various tissues and whether or not the gene is expressed can be characteristically detected by utilizing partial or entire base sequences of the gene.

[0063]

As described above, the gene of the present invention can include the DNA coding for the amino acid sequences shown, for example, in SEQ ID NO 3; however, the gene is not particularly limited to this, but it is needless to say, it also includes a homologue of the gene.

Herein, the homologue of a gene means a series of related genes, which has a sequence homogeneity with the gene of the present invention (or its gene product), and which is recognized as one gene family according to the similarity of the structural characteristic and the biological function as described above, and it is needless to say, it includes an allelic gene of the gene, as well.

[0064]

For example, the gene of the present invention is not limited to the gene having specific base sequences shown with SEQ ID NO 1 or SEQ ID NO 2, but it is also possible to have the base sequences to be selected by combining any codon to each amino acid residue shown with SEQ ID NO 3. The selection of codon can be in accordance with a conventional method, and for example, frequency in use of codon of a host to be utilized can be taken into consideration.

[0065]

Further, as described above, the gene of the present invention even includes DNA hybridizing a DNA having base sequences shown in SEQ ID NO 1 or SEQ ID NO 2 or a part of them. This DNA is a DNA having a certain level or more of homology with the DNA having base sequences shown in SEQ ID NO 1 or SEQ ID NO 2 or a part of them.

[0066]

The above-mentioned DNA having a certain level or more of homology is polynucleotide or a complementary strand polynucleotide having at least 70 % of identity, preferably at least 90 % of identity, or more preferably at least 95 % of or further more preferably 97 % of identity with base sequences shown in SEQ ID NO 1 or 2 or a part of them, amino acid sequences shown in SEQ ID NO 3 or base sequences coding a part of them.

[0067]

To be more specific, for example, the DNA having base sequences shown in SEQ ID NO 1 or SEQ ID NO 2 or a part of the base sequences and the DNA having base sequences to be hybridized can be illustrated under stringent conditions with 0.2 × SSC at 60 degrees C containing 0.1 % SDS or 1 × SSC at 60 degrees C containing 0.1 % SDS.

[0068]

Further, among the DNAs of the present invention, in particular,

DNA having base sequences where one to multiple bases are deleted, added and/or substituted in base sequences or a part of them described in SEQ ID NO 1, and participating in the restoration from sterility of cytoplasmic male sterile individual to fertility;

DNA having base sequences where one to multiple bases are deleted, added and/or substituted in base sequences or a part of them described in SEQ ID NO 2, and participating in the restoration from sterility of cytoplasmic male sterile individual to fertility; and

DNA having base sequences where one to multiple bases are deleted, added and/or substituted in base sequences or a part of them described in SEQ ID NO 3, and participating in the restoration from sterility of cytoplasmic male sterile individual to fertility;

can be produced by an optional method, which is well-known to a person with ordinary skills in the art pertaining to the present invention, such as chemical synthesis, genetic technique or induction of mutation. For example, a mutant gene can be acquired by inducing mutation to DNAs by utilizing the DNA having the base sequences or a part of the base sequences described in SEQ ID NO 1 or 2.

[0069]

As a method to obtain the mutant gene, for example, well-known methods, such as a method using a random mutant, targeted mutant or synthetic gene (see New Genetic Handbook, Supplementary Edition of Experimental Medicine, Yodosha Co., Ltd., 1996) can be used.

Specifically, a method to bring into contact and action with a drug to be mutagens, a method to radiate ultraviolet rays, or a genetic technique can be used upon the DNA having the base sequences described in SEQ ID NO 1 or 2 or part of the base sequences. Since the site-directed mutagenesis, which

is one of genetic techniques, is a technique where a specific variation can be introduced to a specific position, it is useful, and it can be conducted in accordance with the method described in Molecular Cloning Second Edition.

[0070]

(4) Vector containing DNA of the present invention:

The DNA of the present invention can be used as a recombinant vector by recombining into an appropriate vector. The type of vector may be an expression vector or non-expression vector, and it can be selected based upon the purpose.

As a cloning vector, a vector that can be autonomous-replicated among Escherichia coli K12 strains is preferable, and a vector for Escherichia coli expression, such as a pharge vector or plasmid vector that can be used, may be used as a cloning vector. Specifically, it can include ZAP Express [Stratagene, Strategies, 5, 58 (1992)], pBluescrlpt II SK(+) [Nuclelc Acids Research, 17, 9494 (1989)], Lambda ZAP II ((manufactured by Stratagene), λgt10, λgt11 [DNA Cloning, A Practical APPRoach, 1, 49 (1985)], λTriplEx (manufactured by CloneTech), λExCell (manufactured by Pharmacia), pT7T318U (manufactured by Pharmacia), pcD2 [Mo1. Cen. Bio1., 3, 280 (1983)], pMW218 (manufactured by Waco Pure Chemical Industries, Ltd.), pUC118 (manufactured by Takara Shuzo Co., Ltd.), pEG400 [.Bac., 172, 2392 (1990)], and pQE-30 (manufactured by QIAGEN).

[0071]

The expression vector can be selected by considering a combination with a host, and preferably, a vector that enables autonomous replication or incorporation into a chromosome, and that contains a promoter at the position where the gene of the present invention can be transcribed can be used.

When a bacillus is used as a host cell, it is preferable that the expression vector for expression of a DNA is autonomously-replicable in the bacillus; simultaneously, it is preferable that the vector is a recombinant vector comprising a promoter, a liposome-binding sequence, the DNA and the transcription termination sequence. A gene that controls the promoter may be contained.

[0072]

The expression vector for bacillus, for example, pBTrP2, pBTac1, pBTac2 (all are commercially supplied by Boehringer Mannheim GmbH), pKK233-2 (manufactured by Pharmacia), pSE280 (manufactured by Invitrogen), pGEMEX-1 (manufactured by Promega)、pQE-8 (manufactured by QIAGEN), pQE-30 (manufactured by QIAGEN), pKYP10 (Japanese Unexamined Patent Application S58-110600), pKYP200 [Agrc.Biol.Chem., 48, 669 (1984)], PLSA1 [Agrc. Blo1. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci. USA, 82, 4306 (1985)], pBluescrlptII SK+, pBluescriptII SK (-) (manufactured by Stratagene), pTrS30 (FERMBP-5407), pTrS32 (FERM BP-5408), pGEX (manufactured by Pharmacia), pET-3(manufactured by Novagen), pTerm2 (US4686191, US4939094, US5160735), pSupex, pUB110, pTP5, pC194, pUC18 [Gene, 33, 103 (1985)], pUC19 [Gene, 33, 103 (1985)], pSTV28 (manufactured by Takara Shuzo Co., Ltd.), pSTV29 (manufactured by Takara Shuzo Co., Ltd.), pUC118 (manufactured by Takara Shuzo Co., Ltd.), pPA1 (Japanese Unexamined Patent Application S63-233798), pEG400 [J. Bacterio1., 172, 2392 (1990)], pQE-30 (manufactured by QIAGEN) can be illustrated. The promoter for bacillus includes, for example, promoters derived from Escherichia coli or pharge, such as a trp promoter (P trp), a lac promoter (P lac), a PL promoter, a PR promoter or a PSE promoter; a SP01 promoter, a SP02 promoter and a penP promoter.

[0073]

As the expression vector for fermentum, for example, a promoter, such as YEp13 (ATCC37115), YEp24 (ATCC37051), Ycp5O (ATCC37419), pHS19 and pHS15 are exemplified. A promoter for fermentum includes, for example, a PHO5 promoter, a PGK promoter, a GAP promoter, an ADH promoter, a gal1 promoter, a gal10 promoter, a heat shock protein promoter, an MFα1 promoter or a CUP1 promoter.

[0074]

As the expression vector for animal cells, for example, pcDNAI, pcDM8 (commercially supplied from Funakoshi Corporation), pAGE107 [Japanese Unexamined Patent Application H3-22979; Cytotechnology, 3, 133, (1990)], pAS3-3 (Japanese Unexamined Patent Application H2-227075), pCDM8 [Nature, 329, 840, (1987)], pcDNAI/AmP (manufactured by Invitrogen), pREP4 (manufactured by Invitrogen), pAGE103 [J.Blochem., 101, 1307 (1987)], and pAGE210 can be illustrated. The promoter for animal cells includes, for example, a promoter of IE (immediate early) gene of cytomegalovirus (human CMB), an initial promoter of SV40, a retrovirus promoter, a metallothionein promoter, a heat shock promoter and a SRα promoter.

[0075]

As the expression vector for plant cells, for example, pIG121-Hm [Plant Cell Report, 15, 809-814 (1995)], pBI121 [EMBO J. 6, 3901-3907 (1987)], pLAN411 and pLAN421 (Plant Cell Reports 10 (1991), 286-290) can be illustrated. Further, when, in particular, 10 kb or longer DNA fragments are induced into plants, it is desirable to use improved vectors so as to stably maintain and introduce long-chain DNA. For example, pBIBAC2 (Gene 200 (1997) 107-116), pYLTAC7 (PNAS 96 (1999) 6535-6540) and pBIGRZ2 (Bioscience and Industry, 55 (1997), 37-39) are mentioned.

The promoter for plant cells includes, for example, a cauliflower mosaic virus 35S promoter [Mol.Gen.Genet (1990) 220, 389-392]. Furthermore, details of plant transformation will be separately described later.

[0076]

(5) Transformant having DNA of the present invention:

The transformant having DNA of the present invention can be produced by introducing the above-mentioned recombinant vector (preferably, expression vector) to a host.

Specific examples of host cells of bacillus includes microorganisms belonging to Escherichia, Corynebacterium, Brevibacterium, Bacillus, Microbacterium, Serratia, Pseudomonas, Agrobacterium, Alicyclobacillus, Anabaena, Anacystis, Arthrobacter, Azobacter, Chromatium, Erwinia, Methylobacterium, Phormidium, Rhodobacter, Rhodopseudomonas, Rhodospiri11um, Scenedesmun, Streptomyces, Synnecoccus or Zymomonas. A method to introduce a recombinant vector into a cell host includes, for example, a method using calcium ions or the protoplast method.

[0077]

Specific examples of fermentum host can include Saccharomyces cerevisae, Schizosaccharomyces pombe, Kluyveromyces lactis, Trichosporon pu11ulans and Schwanniomyces a11uvius.

As a method for introducing a recombinant vector into fermentum host, any methods for inducing DNA into fermentum can be used, and for example, they can include an electroporation method, a spheroplast method and a lithium acetate method.

[0078]

The animal cell host can include Namalwa cells, COS1 cells, COS7 cells and CHO cells.

As an introduction method of a recombinant vector into animal cells, any method for introducing DNA into animal cells can be used, and for example, the electroporation method, a calcium phosphate method and a lipofection method can be used.

A transformant using plant cells will be described later.

[0079]

(6) Acquisition method for proteins of the present invention:

The acquisition method for the proteins of the present invention is not particularly limited. The proteins of the present invention can be isolated, expressed or synthesized by utilizing a general genetic technique for a person with ordinary skills in the art pertaining to the present invention, based upon information about amino acid sequences obtained by combining the information about the amino acid sequences described in SEQ ID NO 3 or PPR motifs or mitochondrion transfer sequences obtained based upon the amino acid sequences described in SEQ ID NO 3.

For example, the expression can be realized by isolating or synthesizing DNA that codes for the protein of the present invention and by introducing the DNA into cells.

The proteins of the present invention can be obtained, for example, by culturing transformant having the genes of the present invention, by producing and accumulating the proteins of the present invention in the culture, and by collecting the proteins from the culture.

[0080]

The method for culturing the transformant having the genes of the present invention can be in accordance with the normal method used for the culture of hosts.

When the transformant of the present invention is prokaryote, such as Escherichia coli, or eukaryote, such as yeast fungus, the culture medium for

culturing these microorganisms may be either natural or synthetic culture medium, as long as it is the culture medium that contains a carbon source, a nitrogen source or inorganic salts where the microorganisms can be utilized, and where the culture of the transformant can be efficiently conducted. It is preferable to culture the host under aerobic conditions, such as shaking culture or deep ventilation spinner culture, and the culture temperature is 15 degrees C to 40 degrees C, and the culture time is normally for 16 hours to 7 days. During culturing, pH is maintained at 3.0 to 9.0. The pH is adjusted by inorganic or organic acid, an alkali solution, urea, calcium carbonate or ammonia. Further, antibiotics, such as ampicillin or tetracycline, may be added during the culturing as occasions demand.

[0081]

As the culture medium where the transformant obtained using animal cells as a host cell, generally-used RPM11640 culture medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle MEM culture medium [Science, 122, 501 (1952)], DMEM culture medium [Virology, 8, 396 (1959)], 199 culture medium [Proceeding of the Society for the Biological Medicine, 73, 1 (1950)] or culture media where cow's fetus serum is added into these culture media are used. The culture is normally conducted for 1 day to 7 days under conditions of 6 to 8 of pH, 30 degrees C to 40 degrees C of temperature and in the presence of 5 % CO_2. Further, antibiotics, such as Kanamycin or penicillin, may be added to the culture medium during the culturing as occasions demand.

[0082]

As the culture medium for culturing the transformant obtained using plant cells as a host cell, culture media normally used according to plant species, such as MS culture medium or R2P culture medium. The culturing is normally conducted for 1 day to 21 days under conditions at 6 to 8 of pH

and 15 degrees C to 35 degrees C [of temperature]. Further, antibiotics, such as kanamycin or hygromycin, may be added to the culture medium during the culturing as occasions demand.

[0083]

In order to isolate and purify the proteins of the present invention participating in the restoration from sterility of cytoplasmic male sterile individual to fertility from the culture of the transformant, normal protein isolation and purification methods may be used.

For example, when the proteins of the present invention are expressed in the state where they are dissolved into cells, after the culturing, the cells are collected via centrifugal separation and suspended into an aquatic buffer solution, the cells are crushed by an ultrasonic crusher, a French press, a Menton-Gaulin homogenizer or a dyno-mill, and a cell-free extract is obtained. A purified preparation can be obtained from supernatant obtained via the centrifugal separation of the cell-free extract using techniques, such as a normal protein isolation purification method, i.e., a solvent extraction method, a salting-out method with ammonia sulfate, a desalination process, a precipitation method with an organic solvent, an anion exchange chromatography method using a resin, such as diethylaminoethyl (DEAE) sepharose, DIAION HPA-75 (manufactured by Mitsubishi Chemical), a cation exchange chromatography method using a resin, such as S-Sepharose FF (manufactured by Pharmacia), a hydrophobic chromatography using a resin, such as butyl sepharose or phenyl sepharose, a gel filtration method using molecular sieving, an affinity chromatography method, a chromatography focusing method, singularly or in combination.

[0084]

Further, when the proteins form an insolubilized body within cells and it is expressed, similarly the cells are crushed after they are collected, and

after the proteins are collected using a normal method from a precipitation fraction obtained via the centrifugal separation, the insolubilized body of the proteins is solubilized with a protein denaturant. After the solubilized solution is diluted or dialyzed to a solution, which does not contain a protein denaturant or a dilute solution where the concentration of the protein denaturant is low enough not to cause denaturing of the proteins, and after the proteins are constructed to a normal steric structure, a purified preparation can be obtained using the similar isolation and purification method.

[0085]

When the proteins of the present invention or derivatives thereof, such as sugar modified bodies, are secreted to the outside of the cell walls, the proteins or derivatives thereof, such as glycosylation bodies, can be collected from the culture supernatant. In other words, a soluble fraction is acquired by processing the culture using the similar technique, such as centrifugal separation, and a purified preparation can be obtained from the soluble fraction by using the similar isolation and purification method.

[0086]

Further, the proteins of the present invention can be produced using a chemical synthesis method, such as an Fmoc method (fluorenylmethyloxycarbonyl method) or a tBoc method (t-butyloxycarbonyl method). Further, the proteins of the present invention can also be synthesized by utilizing peptide synthesizers by Sowa Trading (manufactured by US Advanced Chem Tech), Perkin Elmer Japan (manufactured by US Perkin Elmer), Amersham Pharmacia Biotech (manufactured by Amersham Pharmacia Biotech), Aloka (manufactured by US Protein Technology Instrument), Kurabo (manufactured by US Synthecell-Vega), Japan PerSeptive Limited (manufactured US PerSeptive), Shimadzu Corporation.

[0087]

(7) Transformant of plants having DNA of the present invention:

The base sequences described in SEQ ID NO 1 are base sequences in the extracted form of plant genome-originated base sequences. These base sequences contain a promoter and terminator required for the gene expression to be operable. The vector to be introduced can clone the genes to a general cloning vector, such as cosmid pWE15 (manufactured by STRATAGENE), in the case of a direct introduction method. In the case of utilizing Agrobacterium, it can be cloned to a general vector for plant transformation, such as pBI121 (manufactured by Clontec).

[0088]

Further, DNA of the base sequences where a part of intron is extracted from this sequence (genomic sequence), DNA of base sequences where almost all of intron is extracted, DNA shown with SEQ ID NO 2 or portions corresponding to the 238th to 2,064th base sequences, or DNA that codes for proteins shown with SEQ ID NO 3 or portions corresponding to 80 to 687 residues may be introduced into plant cells.

[0089]

In addition, promoter and terminator portions may be substituted by a promoter and a terminator that function in the well-known plant cells.

Furthermore, when DNA shown with SEQ ID NO 2 or with the 238th to 2,064th DNA thereof or DNA that codes for the proteins described SEQ ID NO 3 or with portions corresponding to the 80 to 687 residues is introduced into the plant cells, a promoter and a terminator are required other than this DNA. As the general vectors normally used well, pBI121 (manufactured by Clonetec) is mentioned; however, in this vector, a 35S promoter of cauliflower mosaic virus is used for the promoter and a terminator of nopaline synthesis enzyme existing in Ti plasmid of A. tumefacience is used for the terminator.

Further, as the promoter required for the expression, the 35S promoter of cauliflower mosaic virus is not limited to be used but a rbcS promoter widely existing in plants may be used, and it is more preferable to use a type of promoter that is expressed during the growing period of pollen, such as T29 promoter, and it is further preferable to use the original promoter coordinated at the upstream of the gene. The terminator is also not limited to the terminator of the nopaline synthesis enzyme, but a 35S terminator of cauliflower mosaic virus can be used, and it is more preferable to use an original terminator coordinated at the downstream of the gene.

In addition, when the DNA described with the 238th to 2,064th base [sequences] in SEQ ID NO 2 or the DNA that codes for the proteins described in SEQ ID NO 3 or with the portions corresponding to 80 to 687 residues thereof are used for the purpose of restoring from the sterility of cytoplasmic male sterile individual to fertility, a transfer sequence to mitochondria becomes required, in addition to these sequences.

As the transfer sequence to mitochondria, DNA shown with the 1st to 237th base [sequences] in SEQ ID NO 2 or the 1st to 79th amino acid sequences in SEQ ID NO 3, or any other well-known transfer sequences can be utilized.

[0090]

The inventors of the present application produced a vector for plant transformation for the purpose of introducing DNA of the Rf gene containing intron, which is contained from the original promoter to terminator existing in the genome, in the original form, shown in SEQ ID NO 1 in the embodiments described below. After the base sequences shown in SEQ ID NO 1 were cut off by a restricted enzyme from clones comprising a part of contig, they were sub-cloned to appropriate cloning vectors, and then, fragments sub-cloned to the vectors for plant transformation pKM424 and pBIGRZ2 were introduced, and a vector that can introduce the fragments into

plants was obtained. This vector was introduced to Agrobacterium for plant transformation. The DNA fragments are incorporated into the plant genome by infecting this Agrobacterium where the vector is maintained to plants.

[0091]

For the plants where the gene of the present invention is applied, for example, oil crops, such as rapeseed, sunflower, soybean or palm; for example, cereal crops, such as rice, corn or wheat; for example, flowers and ornamental plants, such as tobacco or petunia; and for example, various vegetables, such as tomato, broccoli, cabbage, Chinese cabbage or carrot, can be exemplified.

Among them, genus Brassica plants, such as rapeseed, cabbage, Chinese cabbage or broccoli, and tomatoes are preferable, and rapeseed, cabbage, Chinese cabbage and broccoli are particularly preferable, and rapeseed is the most preferable.

[0092]

In this specification, the transformation plant source includes seeds, germs, nursery plants, callus, cultured cells and plant bodies, and a preferable part may be selected according to a subject plant as normally conducted by a person with ordinary skills in the art pertaining to the present invention, for example, in the case of rapeseed, germ or protoplast; in the case of soybeans, callus or cultured cells; in the case of sunflower, germ; in the case of palm, callus or cultured cells; in the case of rice, germ, callus, cultured cells or protoplast; in the case of corn, germ, nursery plants, callus, cultured cells or protoplast; in the case of wheat, germ, callus or cultured cells; in the case of cabbage or broccoli, germ, callus, cultured cells or protoplast; and in the case of carrot, germ, callus, cultured cells or protoplast.

[0093]

The transformation procedure to plants can be conducted in accordance with a conventional method, and for example, a method where after a vector is once introduced into Agrobacterium, the vector is introduced by infecting the Agrobacterium with plant cells, or another method for directly introducing the vector into cells using an electroporation method, a DEAE dextran method, a calcium phosphate method, a polyethylene glycol method, or a particle gun method can be exemplified.

[0094]

For example, as a preferable gene introduction method in the case of rapeseed, the following methods are exemplified:

Hypocotyl of rapeseed variety sprouted in the MS culture medium containing sugar, such as sucrose, as a carbon source is pre-cultured on the MS culture medium containing 2,4-dichlorophenoxyacetic acid and sucrose. Agrobacterium proliferated in the YEB culture medium is harvested via centrifuge, and re-suspension is conducted in the MS culture medium containing sucrose. After the above-mentioned rapeseed hypocotyl is added to this suspension and the mixture is concussed, the extracted hypocotyl is put back into the original pre-cultured medium and it is co-cultivated for three days, and then, the culture is transferred to a selection medium containing plant hormone, such as zeatin or benzylaminopurine, carbenicillin and kanamycin, and selection is conducted. A reproduced individual can be obtained by culturing the obtained regeneration bud via this process in an elongation culture medium optionally containing plant hormone, such as benzylaminopurine, and consecutively in a rooting culture medium optionally containing plant hormone, such as naphthalene acetic acid or benzylaminopurine), and F1 variety where the fertility is restored can be obtained by breeding this individual with a cms line individual.

[0095]

As described above, it becomes possible to restore from the sterility of the cytoplasmic male sterile individual to the fertility by introducing the DNA of the present invention into plants. Furthermore, for the regenerated individual, the expression can be confirmed by breeding with cms line rapeseed and by investigating the fertility of descendant thereof; however, in the case of transformation using rapeseed having a cms cytoplasm as a material, the pollen fertility can be investigated by transferring a transformant (regenerated individual) where a root is formed as described above into soils containing normal fertilizers and by blooming, and this is preferable because the method is temporally and operationally simple.

[0096]

Further, in the transformation, in the case of transformation as described above by utilizing cells or tissues of rapeseed having the cms cytoplasm, preferably, hypocotyl, cotyledon, leaves, pollen, cultured cells, callus or protoplast, as cells to be used; a plant individual where the pollen fertility is restored can be obtained by transferring the plant body (regenerated individual) obtained with the above-mentioned method to soils containing normal fertilizers and by blooming.

In other words, using cms cells, after the DNA of the present invention is introduced using the above-mentioned gene introduction method to the cms cells and the cells where the DNA is incorporated into a nuclear are selected using resistance to antibiotics or herbicide-tolerant selection markers, such as kanamycin, as an index, a plant body where the DNA is incorporated into the nuclear can be obtained by culturing in the prolongation culture medium or the rooting culture medium. The male sterile character is restored, and this plant body becomes fertile.

[0097]

As a method for detecting the gene participating in the restoration from cytoplasmic male sterility, it can be conducted by confirming such that a quantity of base sequences to be amplified by the primer or a quantity of base sequences to be detected by the probe is one gene or more per genome in the target biological sample using an oligonucleotide primer with 15 mer to 50 mer optionally set from the DNA according to any of Claims 1 to 4 or a probe with at least 15 mer or more comprising entire or a part of the DNA according to any of Claims 1 to 4.

[0098]

The specific confirmation technique includes, for example, the PCR method and the Southern hybridization method, and between them, the PCR method is preferable. These techniques can be conducted in accordance with the method described in Molecular Cloning: A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989 (hereafter, abbreviated as "Molecular Cloning 2nd Ed").

[0099]

In order to confirm that one gene or more exist in one genome, it is necessary to confirm the same degree of amplification using the same number of DNA copies as a template, as a simplified method in the PCR method, and to be more accurate, using the quantification PCR method, this can be confirmed by comparing the amplification quantity of known genes with a quantity of base sequences to be amplified by the primer in the biological sample, using any primer that amplifies known gene, which is known that one gene exists in one genome as internal standard. In the Southern hybridization method, DNA of the fertility restored line plant individual, which is known that one gene of DNA exists in one genome, is compared to

the DNA of a target plant sample, and whether or not the detected DNA quantity is the same or greater.

[0100]

As the primer used for the PCR method, for example, oligonucleotide with 15 mer to 50 mer, which is the same as DNA sequences described in SEQ ID NO 1 or SEQ ID NO 2, or which has complementation, is exemplified.

As the probe used for the Southern hybridization method, the entire area of double-strand DNA or a portion with at least 15 mer or more of DNA sequences described in SEQ ID NO 1 or SEQ ID NO 2, or single-strand DNA or the entire area of complementation or a portion with at least 15 mer or more are exemplified. Further, as described above, DNA having a certain level or more of homology with the base sequences of DNA used as the probe as mentioned above can be exemplified. The 'a certain level or more of homology' herein means, for example, 70 % or more, preferably 80 % or more, more preferably 90 % or more, further preferably 93 % or more, particularly preferably 95 % or more, and the most preferably 97 % or more. Furthermore, as DNA having a certain level or more of homology herein contains both polynucleotide having the homology and complementary strand polynucleotide.

[0101]

The method for detecting genes as described above is not only used for confirmation about whether or not the DNA is incorporated in the transformant, but also used as a means to confirm the presence of Rf gene in the individual where the introduction of Rf gene is attempted due to breeding. If using this method, when the Rf gene is introduced into a cytoplasmic male sterile individual, it is possible to confirm the presence of the Rf gene before blooming. Further, when the Rf gene is introduced into an individual having normal cytoplasm, the fertility of the next generation individual, which was

obtained by breeding pollen at the time of blooming to the cytoplasmic male sterile individual, has to be confirmed; however, the presence of the Rf gene can be confirmed before this stage by using this method. This utilization method is generally referred to as utilization of marker DNA or marker DNA breeding. In the case of Rf gene, utilization as a marker DNA (Rf marker) of the Rf gene can be considered. The Rf marker, as described above, is important using a plant where the Rf gene is introduced by breeding, which is the recombinant where the DNA is introduced or non-recombinant, and breeding the practical variety.

[0102]

Furthermore, in order to confirm whether or not the introduced DNA functions as the Rf gene, it is also possible to confirm the fertility restoration of the transformant; however, this can be conducted using the method mentioned below.

As described above, the Rf gene restores the fertility of a plant by reducing the accumulation within the mitochondria of ORF125 or ORF138, which is a cms causal protein. Therefore, in the mitochondria of the transformant, whether or not the introduced gene is the Rf gene can be confirmed by confirming the reduction in the accumulation of ORF125 or ORF138.

[0103]

The method to confirm the reduction in accumulation of ORF125 or ORF138 protein in the mitochondria is a method to confirm that when ORF125 or ORF138 protein is detected using the Western blotting method in accordance with the conditions described in this specification, a signal amount of an antibody to the mitochondria genome-derived protein to be used as an internal standard, for example, anti-F_1-F_0ATPase (hereafter, abbreviated as ATPA) described in N. Koizuka, et al. Theor Appl Genet, 100:

949-955, 2000 is equal to the cytoplasmic male sterile individual and the transformed individual where the DNA is introduced to a fertility restoration individual or cytoplasmic male sterile individual, and, the accumulation in the transformed individual where the DNA is introduced into the fertility restored individual or the cytoplasmic male sterile individual is reduced by 50 % or more, preferably 60 % or more, and further preferably 80 % or more, compared to the accumulation of ORF125 or ORF138 protein in the cytoplasmic male sterile individual.

[0104]

In actuality, it is observed that in buds of cytoplasmic male sterile radish having ORF125, when the fertility restoring gene Rf is introduced, the accumulation of ORF125 protein is drastically decreased, and it is hardly detected. Further, regarding rapeseed, in the buds of the fertility restored rapeseed where the fertility restoring gene is introduced into the cytoplasmic male sterile rapeseed having cytoplasm ORF125 due to breeding; it is observed that the accumulation of ORF125 protein is reduced by 80 % or more. Further, even in embodiments, in the buds of transformed rapeseed where the DNA is introduced to the cytoplasmic male sterile individual, it is also observed that the accumulation of ORF125 protein is reduced by 80 % or more.

Furthermore, the antibody to ORF125 or ORF138 protein in the method can be obtained by using the general technique as described below. In other words, antiserum can be obtained by immunizing these proteins to animals as an antibody, and an immune globulin G antibody can be purified by using an affinity column where protein A is combined. An antigen to be used can be obtained by purifying proteins from expressed cytoplasmic male sterile plants or cultured cells thereof using a usual method. Further, this antigen can be obtained by connecting ORF125 or ORF138 gene to the expression vector for expressing in Escherichia coli or ferment and by

similarly purifying. In addition, peptide where the entire length or a part of ORF125 or ORF138 is chemically synthesized can be used as an antigen. The antibody to ATPA can also be obtained using a similar technique.

[0105]

In addition, a new hybrid seed production system that does not require any male sterility maintainer line (maintainer line) *[sic.]*, which is necessary for the hybrid seed production, can be produced by specifically and temporarily controlling the expression of the DNA of the present invention, by introducing a part of or all of genes of the present invention along with an induced promoter into cells having cms cytoplasm and having the DNA of the present invention.

[0106]

In other words, the cms line rapeseed is normally sterile, in order to proliferate and maintain the cms line, a maintainer line where cms and Rf do not participate is separately required, and conventionally, three lines of plants, Rf line, cms line and maintainer line, are required for the purpose of production of hybrid seeds; however, because the Rf gene is isolated and identified according to the present invention, on the occasion of the hybrid production, the cms line that enables proliferation and maintenance without the maintainer line can be structured by inducing a promoter by a chemical substance and using a method for controlling the expression of restoring genes.

[0107]

Specifically, a part of or an entire length of the gene of the present invention is incorporated into a promoter induced from the outside, for example, a vector having a promoter with drug sensitivity in an antisense or

sense direction, and cells having the cms cytoplasm and having the DNA of the present invention are transformed using the vector.

As the cells having the cms cytoplasm and having the DNA of the present invention, not only cells having the cms cytoplasm are transformed by the DNA of the present invention in accordance with the above-mentioned method, but cells obtained by breeding the cms line and the Rf line are also usable.

As the inducible promoter, for example, it is known in Japanese Unexamined Patent Application H6-46697, and as a method for vector production and transformation, the similar techniques can be exemplified.

[0108]

Since the transformant, which is a cell having the cms cytoplasm obtained by the above-mentioned method and having the DNA of the present invention, and where a part or entire DNA of the present invention is incorporated along with the inducible promoter, normally does not induce a promoter, the plant shows fertility because of the originally existing Rf genes and the line can be maintained by self-pollinating; however, on the occasion of hybrid production, if a chemical substance having a capability to induce a promoter acts on this plant and the promoter is induced, the expression of the Rf gene is inhibited. Because of this action, the plant becomes male sterile, and this can be used as the cms line at the time of hybrid seed production.

Therefore, if this method is used, since proliferation and maintenance can be conducted by self-pollinating even with the cms line, the maintainer line is no longer required even though three lines used to be conventionally required for the production of hybrid seeds; therefore, it becomes possible to drastically reduce the production cost.

Hereafter, embodiments will be described in further detail; however, the present invention shall not be limited to these embodiments.

[0109]

[Embodiment]

Embodiment 1: Isolation of DNA marker linked to cytoplasmic male sterility restoring gene and preparation of genome map

In order to an isolate fertility restoring gene (Rf gene), first, it is necessary to isolate the DNA marker positioned in the vicinity of the Rf gene and to prepare a genome map showing the gene distance relationship between this DNA marker and the Rf gene. As a starting point, positional cloning of the Rf region was conducted.

[0110]

For the DNA marker isolation method, AFLP was conducted in accordance with an AFLP analysis system I AFLP starter primer kit, based upon the AFLP (amplified fragment length polymorphism) method (Nucleic Acids Research, 1995, Vol.23, No.21 4407-4414). As materials to measure the genetic distance from the marker, approximately 2,100 individuals of F2 aggregation obtained by self-pollinating eight individuals of radish F1 generation, which were [obtained] by breeding one individual of cms line kosena radish (Raphanus sativus cv. Kosena) ((KC2/KA1)-1) and one individual (yuan 10-3) of Rf line Yuanhong radish (Raphanus sativus cv. Yuanhong) in accordance with the method described in N. Koizuka, et al. Theor Appl Genet, 100:949-955 2000, were used. As a result, five markers linked to the positions separated at 0.2 to 0.3 cM of genetic distance at both sides so as to interpose the Rf gene were isolated. A genome map showing the genetic distance between the DNA marker and the Rf gene is shown in Fig. 1, respectively.

[0111]

Embodiment 2: Preparation of contig based upon genome map and analysis of Rf gene

Following the preparation of the genome map, it is necessary to clone the genome DNA corresponding to the position, and to connect the DNA markers interposing the Rf gene. Herein, since the distance between the DNA marker and the Rf gene is apart, the contig of the Rf gene region to cover in between the DNA markers was prepared by connecting multiple clones having the genome DNA fragments.

[0112]

An aggregation of clones having genome DNA fragments is referred to as a genomic library, and we prepared two types of libraries. As a DNA donor, the genome DNA was prepared using the CTAB method (Murray, M. G. and Thompson, W. F. (1980) Nucleic Acids Res., 8, 4321) with Yuanhong radish (Raphanus sativus cv. Yuanhong), which is the same as a parent of the restoration line utilized at the time of production of F_2 aggregation. For the library, a lambda pharge library with 20 kb of average length and 1.5×10^5 of the number of aggregations was prepared using a λDASHII vector (manufactured by STRATAGENE) as a lambda vector. Further, a cosmid library with 40 kb of average length and 5.5×10^4 of the number of aggregations was prepared using a pWEB::TNC vector (manufactured by EPICENTRE TECHNOLOGIES) as a cosmid vector.

[0113]

For the contig production, first, lambda clones were isolated from the prepared lambda library using the DNA markers positioned at both sides of the Rf gene as indexes, using the plaque hybridization method. Further, the cosmid clones were isolated from the cosmid library using the colony hybridization method, and the contig to cover in between the DNA markers

at both sides as shown in Fig. 1 was completed. The cosmid clones NIT7/2 and TO3-2 comprising a part of the contig determined the base sequences by a conventional method.

[0114]

Subsequently, the base sequences of the cosmid clones NIT7/2 and TO3-2 comprising a part of the contig were analyzed using "Genscan" (manufactured by Mitsubishi Space Software), by taking a parameter to Arabidopsis whose all genomes have been recently determined into consideration, because the genome DNA sequences are similar to those of radish. As a result, a promoter portion where the Rf gene seems to be expressed, a structural gene portion containing intron, and a terminator portion were discovered. In addition, the genes in the form to be translated into protein where intron is removed and the amino acid sequence to the genes were obtained.

[0115]

Embodiment 3: Sub-cloning of genomic DNA region

HpaI-SwaI fragments of DNA comprising 1 to 8,553 base sequences described in SEQ ID NO 1 sufficiently containing the terminator from the promoter assumed by "Genscan" were separated from the vector by gel electrophoresis using agarose for fragment collection (manufactured by FMC). A gel containing DNA fragments was digested by the gel degrading enzyme (manufactured by Epicentre Technologies), and DNA was collected. In addition, cloning fragments where the obtained fragments were cut by a restriction enzyme BamHI were obtained. These DNA fragments were sub-cloned to a pGEM-T easy vector (manufactured by Promega), and cds6BT/pGEM-T easy [vector] was obtained. Hereafter, details will be described.

[0116]

Addition of 1 μg of NIT7/2 cosmid DNA and 10 units of restriction enzyme HpaI (manufactured by Takara Shuzo) into 100 μl of 1×K restriction enzyme buffer solution (20 mM Tris-HCl (pH8.5), 10 mM $MgCl_2$, 1 mM dithiothreitol and 100 mM KCl) was conducted, and the mixture was heated for 1 hour at 37 degrees C.

[0117]

After heating, after 10 μl of 3M sodium acetate (pH 5.6) and 250 μl of ethanol were added and agitated, the mixture was cooled down at -80 degrees C for 5 minutes, and this was centrifuged at 15,000 rpm and 4 degrees C for 15 minutes. A supernatant is removed, and the precipitation was dried for 5 minutes using a centrifugal vacuum drier. Addition of 89 μl of sterilized water into the collected DNA precipitation was conducted and the precipitation was dissolved.

[0118]

Addition of 10 μl 10×H restriction enzyme buffer solution (500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 10 mM dithiothreitol and 1000 mM NaCl), 1 μl of 10 unit/μl restriction enzyme SwaI (manufactured by Takara Shuzo) into the dissolved DNA solution was conducted, and [the mixture] was heated at 25 degrees C for 1 hour. Addition of 11 μl of 10x loading buffer solution (1 % SDS, 50 % Glycetrol and 0.05 % Bromophenol Blue) was conducted.

[0119]

After mixing 1.2 g of low melting-point agarose, SeaPlaqueGTG agarose (manufactured by FMC) and 150 ml of 1 × TAE (40 mM Tris-acetate, 1 mM EDTA) buffer solution, [the mixture] was heated to 100 degrees C and agarose was dissolved, and it was cooled down to 45 degrees C while agitating. A comb with 30 mm of width × 1 mm of thickness was placed on a gel tray of

14 cm × 15 cm, and the cooled gel was poured into the gel tray and the gel was hardened. The DNA where loading dye was added was poured into the gel comb, and electrophoresis was conducted at 1 × TAE and 30 V/30 cm of voltage for 18 hours.

[0120]

The electrophoresis gel was transferred into a 0.5 μg/ml ethidium bromide/ 1 × TAE solution, and dyeing was conducted. The gel was placed on a transilluminator where 365 nm of long-wave ultraviolet rays was irradiated, and target 8,546 bp of fragments were cut using a sterilized surgical knife. In addition, the gel was chopped to be approximately 1 mm square of fragments, and transferred into pre-weighed 2 ml microtube, and the gel was weighed.

[0121]

Addition of 1 μl of 50x GELase buffer (2M Bis-Tris (pH6.0), 2M NaCl) into 50 mg of gel was conducted. The tube containing the gel was placed into a dry heat block heated to 68 degrees C; the tube was sometimes agitated up and down; the gel was heated for 10 minutes and was completely dissolved. This tube was transferred to a dry heat block at 45 degrees C, and the tube was sometimes agitated up and down, and the gel was heated for 5 minutes. Addition of 1 unit of GELase (manufactured by Epicentre Technologies) into 200 mg of gel weight in this tube was conducted, and the tube was sometimes agitated up and down and the gel was heated for 30 minutes.

[0122]

Addition of 1/3 of gel volume of 10M ammonium acetate (pH 7.0) was conducted and [the tube] was agitated, and centrifuge was conducted at 15,000 rpm for 5 minutes. The supernatant was transferred to a new 2-ml microtube, and 2 volumes of ethanol were added to the supernatant. After

the tube was agitated, it was centrifuged at 15,000 rpm and 4 degrees C for 20 minutes. The supernatant was removed, and 1 ml of 70 % ethanol was further added quietly, and it was centrifuged at 15,000 rpm and 4 degrees C for 5 minutes. The supernatant was removed, and the precipitation was dried for 5 minutes using a centrifugal vacuum drier. Addition of 20 μl of TE buffer solution (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) into the precipitation was conducted, and the precipitation was completely dissolved, and the DNA fragments were collected.

[0123]

Addition of 10 μl of 10×K restriction enzyme buffer solution (200 mM Tris-HCl (pH 8.5), 100 mM $MgCl_2$, 10 mM dithiothreitol, 1,000 mM KCl), 68 μl of dH_2O, 2 μl of 10 unit/μl restriction enzyme BamHI (manufactured by Takara Shuzo) into the collected 20 μl of DNA solution was conducted, and the mixture was heated at 30 degrees C for 1 hour. After heating, 10 μl of 3M sodium acetate (pH 5.6) and 250 μl of ethanol were added and agitated, and then, the mixture was cooled at -80 degrees C for 5 minutes and centrifuged at 15,000 rpm and 4 degrees C for 15 minutes. The supernatant was removed, and 1 ml of 70 % ethanol was further added quietly, and then, the mixture was centrifuged at 15,000 rpm and 4 degrees C for 5 minutes. The supernatant was removed, and the precipitation was dried for 5 minutes using the centrifugal vacuum drier. Addition of 20 μl of sterilized water to the collected DNA precipitation, and the precipitation was dissolved. After 55 μl of sterilized water, 10 μl of 10 % PCR buffer solution (100 mM Tris-HCl (pH 8.3), 500mM KCl), 6 μl of 25 mM $MgCl_2$, 8 μl of 2.5 mM dNTP mix and 1 μl of 5 unit/μl rTaq DNA polymerase (manufactured by Takara Shuzo) and the mixture was mixed, it was heated at 72 degrees C for 30 minutes and dATP was added to 3' terminal.

[0124]

The reaction liquid was transferred to an ultrafiltration filter unit: Microcon-50 (manufactured by Millipore), and the reaction liquid was centrifuged at 5,000 rpm and 4 degrees C for 20 minutes. Water in the trap was disposed and 100 µl of sterilized water was added, and the mixture was centrifuged at 5,000 rpm and 4 degrees C for 20 minutes. Addition of 20 µl of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) was conducted, and the filter unit was removed and the posture was changed upside down, and it was mounted to a new microtube. The microtube was centrifuged at 3,000 rpm and 4 degrees C for 5 minutes.

[0125]

After 1 µl of 50 ng/µl pGEM-T easy vector (manufactured by Promega) and 6 µl of DNA Ligation Kit Ver.2 (manufactured by Takara Shuzo) solution I were mixed into 5 µl of purified DNA obtained by the above-mentioned method, the mixture was incubated at 16 degrees C for 30 minutes.

[0126]

The reaction liquid was transferred to the ultrafiltration filter unit: Microcon-50 (manufactured by Millipore) along with 100 µl of sterilized water, and the filter unit was centrifuged at 5,000 rpm and 4 degrees C for 20 minutes. Water in the trap was disposed and 100 µl of sterilized water was added again, and the filter unit was centrifuged at 5,000 rpm and 4 degrees C for 20 minutes. The filter unit was removed and the posture was changed upside down, and it was mounted to a new microtube. The microtube was centrifuged at 3,000 rpm and 4 degrees C for 5 minutes, and DNA in the filter unit was collected.

[0127]

The collected DNA was set up together with the tube on the ice and cooled. Placement of 30 µl of Escherichia coli DH10B for electroporation (manufactured by Gibco BRL) into a tube was conducted, and the tube was slightly mixed. The Escherichia coli mixed with DNA was transferred to a pre-iced cuvette for electroporation (1 mm of electrode intervals) (manufactured by USA Scientific Plastics). Electroporation was conducted under 1.25 kv, 129 Ω and 50 µF of conditions using Electro Cell Manipulator 600 (manufactured by BTX), and immediately after that, 500 µl of SOC culture medium (manufactured by Gibco BRL) warmed to 37 degrees C was added to the cuvette. The Escherichia coli were transferred to a 10-ml culturing tube, and it was shaken & cultured at 37 degrees C for 1 hour. The cultured Escherichia coli were spread to a LB agar medium (1 % Bacto-Tryptone, 0.5 % Bacto-Yeast Extract, 1 % NaCl and 1.5 % Bacto-Agar) where 100 µg/ml of Ampiciline (manufactured by Waco Pure Chemical Industries), 20 µg/ml X-Gal (manufactured by Takara Shuzo) and 1 mM IPTG (manufactured by Takara Shuzo), and it was cultured at 37 degrees C for 18 hours or longer.

[0128]

A white colony appeared on the agar medium and it was cultured on 2 ml of the LB culture medium where 100 µg/ml of AmpiciIlin was added at 37 degrees C for 18 hours or longer. Plasmid DNA was extracted from the cultured Escherichia coli using a usual method. It was confirmed that target fragments were cloned in the plasmid DNA via cutting with a restriction enzyme EcoRI (manufactured by Takara Shuzo), and cds6BT/pGEM-T easy [vectors] were obtained.

[0129]

Each of Escherichia coli DH10B where cds6BT/pGEM-T easy [vectors] obtained using the above-mentioned method was maintained was cultured on 100 ml of the LB agar medium where 100 μg/ml AmpiciIlin was added at 37 degrees C for 18 hours. According to the alkali SDS method, the culture was purified using a Qiagen Midi kit (manufactured by Qiagen).

[0130]

Embodiment 4-1: Production of vector for plant transformation (1)

After cds6BT/pGEM-Teasy [vectors] were cut by the restriction enzyme EcoRI, they were separated from a vector by the gel electrophoresis using agarose for fragment collection, and the collected DNA fragments were cloned to the EcoRI section of vector pKM424 for plant transformation (a vector where fragments of CaMV35S promoter: GUS gene: NOS terminator were added is pLAN421 (Plant Cell Reports 10 (1991) 286-290 vector), and a vector for plant transformation cds6BT/pKM424 were obtained. Hereafter, details will be described.

[0131]

Addition of 1 μg of cds6BT/pGEM-T easy DNA and 10 units of restriction EcoRI (manufactured by Takara Shuzo) into 100 μl of 1×H restriction enzyme buffer solution (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol and 100 mM NaCl) was conducted, and the mixture was heated at 37 degrees C for 1 hour.

Hereafter, the EcoRI fragments containing cds6BT were separated and collected from cds6BT/pGEM-T easy using the similar method where the HpaI-SwaI fragments were extracted.

[0132]

Addition of 1 μg of vector for plant transformation pKM424 and 10 units of restriction enzyme EcoRI (manufactured by Takara Shuzo) into 100 μl of 1×H restriction enzyme buffer solution (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol and 100 mM NaCl) was conducted, and the mixture was heated at 37 degrees C for 1 hour. After heating, 100 μl of 1M Tris-HCl (pH 8.0) and 1 unit of Bacterial Alkaline Phosphatase (manufactured by Takara Shuzo) were added and mixed, and then, the mixture was heated at 50 degrees C for 1 hour and dephosphorylated.

[0133]

Phenol chloroform saturated with 200 μl of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) was added, and the mixture was intensely agitated. After centrifuging at 15,000 rpm for 5 minutes, the supernatant was transferred to a new tube. The similar operation was repeated once more, and a protein was removed. After 20 μl of 3M sodium acetate (pH 5.6) and 500 μl of ethanol were added and agitated, the mixture was cooled at -80 degrees C for 5 minutes and centrifuged at 15,000 rpm and 4 degrees for 15 minutes. The supernatant was removed, and 1 ml of 70 % ethanol was further added, and the mixture was centrifuged at 15,000 rpm and 4 degrees C for 5 minutes. The supernatant was removed, and the precipitation was dried for 5 minutes using a centrifugal vacuum drier. Addition of 100 μl of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) into the precipitation was conducted and the precipitation was completely dissolved, and the concentration was adjusted to 10 ng/μl.

[0134]

After 10 μl of purified EcoRI fragments, 1 μl of dephosphorylated pKM424 vector and 11 μl of DNA ligation Kit Ver.2 (manufactured by Takara

Shuzo) solution were mixed, the mixture was incubated at 16 degrees C for 30 minutes.

[0135]

The above-mentioned reaction liquid was transferred to the ultrafiltration filter unit: Microcon-50 (manufactured by Millipore) along with 100 µl of sterilized water, and the mixture was centrifuged at 5,000 rpm and 4 degrees C for 20 minutes. Water in a trap was disposd and 100 µl of sterilized water was added again, and the mixture was centrifuged at 5,000 rpm and 4 degrees C for 20 minutes. The filter unit was removed, and the posture was changed upside down and mounted to a new microtube.

The mixture was centrifuged at 3,000 rpm and 4 degrees C for 5 minutes, and DNA in the filter unit was collected.

[0136]

The collected DNA was set up together with the tube on the ice and cooled. Placement of 30 µl of Escherichia coli DH10B for electroporation (manufactured by Gibco BRL) into a tube was conducted, and the tube was slightly mixed. The Escherichia coli mixed with DNA were transferred to a pre-iced cuvette for electroporation (1 mm of electrode intervals) (manufactured by USA Scientific Plastics). Electroporation was conducted under 1.25 kv, 129 Ω and 50 µF of conditions using Electro Cell Manipulator 600 (manufactured by BTX), and immediately after that, 500 µl of SOC culture medium (manufactured by Gibco BRL) warmed to 37 degrees C was added to the cuvette. The Escherichia coli were transferred to a 10-ml culturing tube, and it was shaken & cultured at 37 degrees C for 1 hour. The cultured Escherichia coli was spread to a LB agar medium (1 % Bacto-Tryptone, 0.5 % Bacto-Yeast Extract, 1 % NaCl and 1.5 % Bacto-Agar) where 50 µg/ml of Spectinomycin (manufactured by Sigma) was added, and it was cultured at 37 degrees C for 18 hours or longer.

[0137]

A white colony appeared on the agar medium and it was cultured on 2 ml of the LB culture medium where 50 μg/ml of Spectinomycin was added at 37 degrees C for 18 hours or longer. Plasmid DNA was extracted from the cultured Escherichia coli using a usual method. It was confirmed that target fragments were cloned in the plasmid DNA via cutting with a restriction enzyme HindIII (manufactured by Takara Shuzo), and cds6BT/pKM424 were obtained.

Escherichia coli DH10B where cds6BT/pKM424 were maintained using the above-mentioned method were cultured on 250 ml of the LB agar medium where 50 μg/ml Spectinomycin was added at 37 degrees C for 18 hours. According to the alkali SDS method, the culture was purified using a Qiagen Midi kit (manufactured by Qiagen).

[0138]

Embodiment 4-2: Production of vector for plant transformation (2)

After lambda clone CHI (see Fig. 2, cloning fragment length: approximately 17 kb) where the base sequences described in SEQ ID NO 1 were sufficiently maintained was cut by the restriction enzyme NotI (manufactured by Takara Shuzo) existing in the multiple cloning sections, it was separated from a vector by the gel electrophoresis using agarose for fragment collection, and the collected DNA fragments were cloned to the NotI section of vector pBIGRZ2 for plant transformation (Bioscience and Industry 55 (1997) 37-39), and a vector for plant transformation CHI/pBIGRZ2 was obtained. Hereafter, details will be described.

[0139]

Addition of 1 μg of lambda clone CHI DNA and 10 units of restriction NotI (manufactured by Takara Shuzo) into 100 μl of 1×H restriction enzyme buffer solution (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol,

100 mM NaCl, 0.01 % BSA and 0.01 % TritonX-100) was conducted, and the mixture was heated at 37 degrees C for 1 hour. Hereafter, the NotI fragments of the lambda clone CHI were separated and collected using the similar method where the HpaI-SwaI fragments were extracted.

[0140]

Addition of 1 μg of vector for plant transformation pBIGRZ2 and 10 units of restriction enzyme NotI (manufactured by Takara Shuzo) into 100 μl of 1×H restriction enzyme buffer solution (50 mM Tris-HCl (pH7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 0.01 % BSA and 0.01 % TritonX-100) was conducted, and the mixture was heated at 37 degrees C for 1 hour. After heating, 100 μl of 1M Tris-HCl (pH 8.0) and 1 unit of Bacterial Alkaline Phosphatase (manufactured by Takara Shuzo) were added and mixed, and then, the mixture was heated at 50 degrees C for 1 hour and dephosphorylated.

[0141]

Phenol chloroform saturated with 200 μl of TE buffer solution (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA) was added, and the mixture was intensely agitated. After centrifuging at 15,000 rpm for 5 minutes, the supernatant was transferred to a new tube. The similar operation was repeated once more, and a protein was removed. After 20 μl of 3M sodium acetate (pH 5.6) and 500 μl of ethanol were added and agitated, the mixture was cooled at -80 degrees C for 5 minutes and centrifuged at 15,000 rpm and 4 degrees for 15 minutes. The supernatant was removed, and 1 ml of 70 % ethanol was further added, and the mixture was centrifuged at 15,000 rpm and 4 degrees C for 5 minutes. The supernatant was removed, and the precipitation was dried for 5 minutes using a centrifugal vacuum drier. Addition of 100 μl of TE buffer solution (10 mM Tris-HCl (pH8.0) and 1 mM

EDTA) to the precipitation was conducted and the precipitation was completely dissolved, and the concentration was adjusted to 10 ng/μl.

[0142]

After 10 μl of purified NotI fragments, 1 μl of dephosphorylated pBIGRZ2 vector and 11 μl of DNA ligation Kit Ver.2 (manufactured by Takara Shuzo) solution I were mixed, the mixture was incubated at 16 degrees C for 30 minutes.

The above-mentioned reaction liquid was transferred to the ultrafiltration filter unit: Microcon-50 (manufactured by Millipore) along with 100 μl of sterilized water, and the mixture was centrifuged at 5,000 rpm and 4 degrees C for 20 minutes. Water in a trap was disposed and 100 μl of sterilized water was added again, and the mixture was centrifuged at 5,000 rpm and 4 degrees C for 20 minutes. The filter unit was removed, and the posture was changed upside down and mounted to a new microtube. The mixture was centrifuged at 3,000 rpm and 4 degrees C for 5 minutes, and DNA in the filter unit was collected.

[0143]

The collected DNA was set up together with the tube on the ice and cooled. Placement of 30 μl of Escherichia coli DH10B for electroporation (manufactured by Gibco BRL) into a tube was conducted, and the tube was slightly mixed. The Escherichia coli mixed with DNA were transferred to a pre-iced cuvette for electroporation (1 mm of electrode intervals) (manufactured by USA Scientific Plastics). Electroporation was conducted under 1.25 kv, 129 Ω and 50 μF of conditions using Electro Cell Manipulator 600 (manufactured by BTX), and immediately after that, 500 μl of SOC culture medium (manufactured by Gibco BRL) warmed to 37 degrees C was added to the cuvette. The Escherichia coli were transferred to a 10-ml culturing tube, and it was shaken & cultured at 37 degrees C for 1 hour. The

cultured Escherichia coli were spread to a LB agar medium (1 % Bacto-Tryptone, 0.5 % Bacto-Yeast Extract, 1 % NaCl, 1.5 % Bacto-Agar) where 25 μg/ml of Kanamycin (manufactured by Wako Pure Chemical Industries) was added, and they were cultured at 37 degrees C for 18 hours or longer.

[0144]

A colony appeared on the agar medium and it was cultured on 2 ml of the LB culture medium where 25 μg/ml of kanamycin was added at 37 degrees C for 18 hours or longer. Plasmid DNA was extracted from the cultured Escherichia coli using a usual method. It was confirmed that target fragments were cloned in the plasmid DNA via cutting with a restriction enzyme HindIII (manufactured by Takara Shuzo), and CHI/pBIGRZ2 was obtained.

Escherichia coli DH10B where CHI/pBIGRZ2 obtained using the above-mentioned method were maintained were cultured on 250 ml of the LB agar medium where 25 μg/ml kanamycin was added at 37 degrees C for 18 hours. According to the alkali SDS method, the culture was purified using a Qiagen Midi kit (manufactured by Qiagen).

[0145]

Embodiment 5: Introduction of Agrobacterium to vector for plant transformation

Competent cells of Agrobacterium were prepared, and the cds6BT/pKM424 vectors and the CHI/pBIGRZ2 vectors obtained in Embodiments 4-1 and 4-2 were introduced into the prepared Agrobacterium EHA101 for plant transformation, respectively. Hereafter, the details will be described.

[0146]

The competent cells for electroporation of Agrobacterium EHA101 were prepared using the method mentioned below. Agrobacterium EHA101 was streaked over the LB agar culture medium where 50 μg/ml kanamycin (manufactured by Wako Pure Chemical Industries) and 25 μg/ml Chloramphenicol (manufactured by Wako Pure Chemical Industries), and [Agrobacterium EHA101] was cultured at 28 degrees for 24 hours or longer, and a single colony was obtained. The LB culture medium where 50 μg/ml kanamycin and 25 μg/ml Chloramphenicol were added was placed into a 50-ml centrifuging tube and a colony with approximately 1 mm of diameter was inoculated, and the centrifuging tube was shaken and cultured at 28 degrees C for 40 hours. The culture solution was centrifuged at 1,500×g and 4 degrees C, and harvest was conducted. Placement of 40 ml of iced sterilized 10 % glycerol to the tube where the supernatant was removed was conducted and a fungus body was re-suspended, and the tube was centrifuged and harvested at 1,500 × g and 4 degrees C. This operation was repeated twice. Addition of 500 μl of iced sterilized 10 % glycerol into the obtained fungus body was conducted, and the mixture was re-suspended. Divided injection of 100 μl each of fungus body into the sterilized microtubes was conducted, and after these were frozen with liquid nitrogen, they were stored in an 80-degrees C freezer.

[0147]

The competent cells for electroporation of Agrobacterium EHA101 were melted on iced water. Placement of 40 μl of electrocompetent cells into pre-cooled 1.5 ml tube was conducted, and 100 ng of cds6BT/pKM424 or CHI/pBIGRZ2 plasmid DNA was added and slightly mixed.

[0148]

Agrobacterium mixed with DNA was transferred to a pre-iced cuvette (manufactured by USA Scientific Plastics) for electroporation (electrode intervals: 1 mm). Electroporation was conducted under conditions of 1.44 kv, 129 Ω and 50 μF using Electro Cell Manipulator 600 (manufactured by BTX), and immediately after that, 500 μl of SOC culture medium (manufactured by Gibco BRL) was added. Agrobacterium was transferred to a 10-ml culturing tube, and the tube was shaken and cultured at 30 degrees C for 1 hour.

Cultured Agrobacterium where a cds6BT/pKM424 were introduced was spread over the LB agar culture medium (1 % Bacto-Tryptone, 0.5 % Bacto-Yeast Extract, 1 % NaCl and 1.5 % Bacto-Agar) where 50 μg/ml kanamycin (manufactured by Wako Pure Chemical Industries), 25 μg/ml Chloramphenicol (manufactured by Wako Pure Chemical Industries), 50 μg/ml Spectinomycin (manufactured by Sigma) and 2.5μg/ml Tetracycline (manufactured by Sigma) were added, and it was cultured at 28 degrees C for 24 hours or longer.

Cultured Agrobacterium where a CHI/pBIGRZ2 vectors were introduced was spread over the LB agar culture medium where 50 μg/ml kanamycin (manufactured by Wako Pure Chemical Industries), 25 μg/ml Chloramphenicol (manufactured by Wako Pure Chemical Industries) and 30 μg/ml Hygromycin (manufactured by Sigma) were added, and it was cultured at 28 degrees C for 24 hours or longer.

[0149]

A colony appeared on the agar culture medium and it was cultured on 2 ml of LB culture medium where antibiotics adapted to each vector were added at 30 degrees C for 24 hours or longer. Plasmid DNA was extracted from the cultured Agrobacterium using a usual method, and it was confirmed that cds6BT/pKM424 vectors or CHI/pBIGRZ2 vectors were introduced by cutting with the restriction enzyme HindIII (manufactured by Takara Shuzo).

For the confirmed clones, after an equal amount of sterilized 80 % glycerol was added to the culture solution, which was cultured for 24 hours, and they were mixed; they were stored at -80 degrees C and used for rapeseed transformation.

[0150]

Embodiment 6: Production of rapeseed transformant

The transformation to rapeseed was conducted as follows: CMS rapeseed (SW18) seeds having radish-derived cms causal genes ORF125 were sterilized with a 10 % hydrochlorous acid solution, and they were sprouted on the MS culture medium (T. Murashige and F. Skoog Physiol. Plant. 15:485, 1962) not containing hormone. Only hypocotyl parts were cut off from seedling where 7 days to 14 days passed after sprout, they were cut into 3 to 5 mm of length, and they were pre-cultured on the MS culture medium (M5519 provided by Sigma) + 3 % sucrose + 1 mg/l 2.4-D and 0.4 % agarose (Sigma, type I) at 23 degrees C for 12 to 16 hours. At this time, for the protection culture, cocultivation with tobacco-derived cell line BY-2 was conducted.

[0151]

In the meantime, Agrobacterium containing CHI/pBIGRZ2 was cultured at 28 degrees C for 8 to 48 hours, and was proliferated to approximately OD_{600} = 1.0. The fungus body of Agrobacterium was suspended in a liquid MS hormone-free culture medium. The cut hypocotyl and this Agrobacterium solution were mixed, and the mixture was cocultivated for approximately 20 minutes. After the cocultivation, the hypocotyl where Agrobacterium was removed with a paper filter was cultured, for example, in a culture medium with MS basic culture medium + B5 vitamin (Sigma, M0404) + 3 % sucrose + 1 mg/l 2,4-D, for 2 days and infected. After the infection, the hypocotyl was transplanted to a sterile culture

medium where an antibiotic: carbenicillin (geopen by Pfizer or carbenicillin disodium salts by GIBCO-BRL) was added with 500 mg/l of concentration into MS basic culture medium + B5 vitamin + 3 % sucrose + 1 mg/l 2,4-D, and Agrobacterium was removed.

[0152]

After five days to one week were passed with the above-mentioned sterile culture medium, the hypocotyl was added to MS basic culture medium + B5 vitamin + 1 % sucrose + 3mg/l benzylaminopurine + 500mg/l carvenicillin, and it was cultured on a culture medium where 5 mg/l silver nitrate and 5 to 30 mg/l kanamycin for selection (kanamycin sulfate salt, Nacalai Tesque) were added, for 14 days to 21 days. At this time, since green callus might appear, they were immediately transplanted to a culture medium in the next step.

[0153]

As the culture medium in the next step, for example, there is a selection medium containing MS culture medium (Sigma, M5519) + 1 % sucrose + 3 mg/l benzylaminopurine + 1 mg/l zeatin + 500 mg/l carbenicillin + 5 to 30 mg/l kanamycin. The hypocotyl where the callus was formed from the incision was transplanted to this culture medium and cultured at 23 degrees C for 3 weeks, and then, 3 to 5 times of transplants were repeated every 3 weeks until the green callus appeared.

[0154]

The green callus was cut off from the hypocotyl immediately as soon as it was discovered, and it was transferred to the culture medium with the same composition. After that, when only green portions were cut off and joined, adventitious buds were formed with 1 % to 30 % of probability. After the adventitious buds were transferred to B5 basic culture medium (Sigma,

G5893) + 3 % sucrose + 1 mg/l benzylaminopurine and cultivated thereafter, they were rooted in the culture medium containing MS culture medium (Sigma, M5519) + 3 % sucrose + 0.1 mg/l naphthalene acid + 0.1 mg/l benzylaminopurine.

[0155]

Embodiment 7: Analysis of transformant (detection of introduced DNA)

One leaf was extracted from one transformation individual where buds were formed obtained in Embodiment 6, and DNA was isolated using a DNA isolation kit (DNeasy plant mini) by QIAGEN.

Three sections (sections *a*, *b* and *c*) of the introduced DNA fragments were detected using the PCR method (results are shown in Fig. 3). The section *a* contains 568bp from 3,186bp to 3,753bp of base sequences in SEQ ID NO 1, and [5'-GAAGCAAAAAAGAAAACGAGCAGAG-3'] (SEQ ID NO 4) and [5'-CCAAAAATCCGAAATCCGAATAGAC-3'] (SEQ ID NO 5) were used as a forward primer and a reverse primer, respectively. The section *b* contains 244bp from 4,869bp to 5,112bp of base sequences in SEQ ID NO 1, and [5'-CTCGGCTCTGGGTTTAGTGA-3'] (SEQ ID NO 6) and [5'-TCCACAAACCCTAGCCAACA-3'] (SEQ ID NO 7) were used as a forward primer and a reverse primer, respectively. The section *c* contains 485bp from 7,766bp to 8,250bp of base sequences in SEQ ID NO 1, and [5'-GCTTATGCTTCTCTGGTTCGCCTC-3'] (SEQ ID NO 8) and [5'-CTCAGTTTTCGTCACCTTACACAATGC-3'] (SEQ ID NO 9) were used as a forward primer and a reverse primer, respectively.

[0156]

After 12.1 μl of sterilized water, 2 μl of 10 × PCR buffer solution (100 mM Tris-HCl (pH8.3), 500 mM KCl), 1.2 μl of 25 mM $MgCl_2$, 1.6 μl of 2.5 mM dNTP mix, 1 μl of 10 μM forward primer solution of each section, 1 μl of 10 μM reverse primer solution of each section, 0.1 μl of 5 unit/μl rTaq DNA

polymerase (manufactured by Takara Shuzo) were added to 1 μl of transformant DNA solution (50 ng/μl) and mixed, DNA was amplified by repeating a cycle of 94 degrees for 40 seconds, 55 degrees C for 30 seconds and 72 degrees for 1 minute, 33 times. For a thermal cycler, UNOII (manufactured by Biometra) was used. After the reaction was completed, an amplified product was confirmed by gel electrophoresis of 4 % Nusive 3:1 Agarose (manufactured by FMC)/ 1×TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) buffer solution (see Fig. 3).

[0157]

As a result, it was ascertained that the section *a* was not introduced into this transformed rapeseed. An amplified product with the same size as the positive control was obtained at the remaining two sections (the sections *b* and *c*), respectively, and it was confirmed that the DNA was incorporated into the transformed rapeseed.

[0158]

Embodiment 8: Analysis of transformant (confirmation of decrease in cms causal protein ORF125 accumulation)

One bud from the same individual in Embodiment 7, and a decrease of ORF125, which is CMS protein) was analyzed by the Western blotting method. The results are shown in Fig. 4.

[0159]

(1) Protein extraction from transformed individual

The Western blotting method, which is a protein extraction method, was conducted based upon the method by N. Koizuka et al. (Theor Appl Genet (2000) 100:949-955).

Specifically, one bud (1 mm of length) of the obtained transformed rapeseed and 100 μl of iced protein extracted buffer solution (50 mM Tris-HCl (pH7.5), 2 % (W/V) SDS) were placed into an iced mortar, and they were

ground by a pestle. This liquid was transferred to a micro-centrifugal tube, and it was centrifuged at 15,000 rpm and 4 degrees C for 15 minutes. After centrifuging, a supernatant solution was transferred to a new micro-centrifugal tube, and it was heated at 100 degrees for 5 minutes. The supernatant solution was centrifuged at 15,000 rpm and 4 degrees C for 15 minutes again, and a supernatant solution was transferred to a new micro-centrifugal tube, and this solution was regarded as a SDS soluble protein solution. The concentration of the SDS soluble protein solution was measured using a protein quantitative kit (manufactured by Bio-rad) by a Bradford method. Along with this, the SDS soluble protein solution was similarly extracted from buds from rapeseed of a cytoplasmic male sterile line and rapeseed of fertility restored line, as well, and the concentration was measured, respectively.

[0160]

(2) Isolation of protein by SDS-PAGE method and transcription to PVDF film: Western blotting

Placement of 15 μg of the SDS soluble protein per lane onto a 10 % SDS polyacrylic amide gel with 7 cm × 10 cm square was conducted, and the protein was isolated by electrophoresis. In addition, for the purpose of comparison of ORF125 protein accumulation, a diluted line of the cytoplasmic male sterile line rapeseed was similarly placed and isolated. The electrophoresis conditions were for 1 hour with 10 mA and for 1 hour with 15 mA. After the electrophoresis, the protein in the polyacrylic amide gel was transcribed onto a PVDF film (manufactured by Millipore) under the condition with 100 mA for 1 hour.

[0161]

(3) Detection of protein using antibody: Western blotting

The PVDF film where the protein was transcribed was divided into upper and lower two films, and they were transferred to 10 ml of blocking solution (20 mM Tris-HCl (pH 7.5), 500 mM NaCl, 0.05 % Tween20 and 5 % skim milk), and they were shaken for 1 hour and blocked. ATPA as a control of mitochondria protein mass and ORF125, which is a cytoplasmic male sterility related protein, were detected from the upper PVDF film and the lower PVDF film, respectively. The PVDF films were transferred to 10 ml of primary antibody reaction liquid (100 µl of ATPA monoclonal antibody was added for ATPA detection, and rabbit antiserum to 2 µl of ORF125 was added for ORF125 detection into 10 ml of blocking solution (M.Iwabuchi et al. Plant Molecular Biology (1999) 39: 183-188)) was conducted, and they were shaken for 18 hours. The PVDF films were transferred to 100 ml of TTBS (20 mM Tris-HCl (pH 7.5), 500 mM NaCl and 0.05 % Tween20) and shaken for 10 minutes. This operation was repeated three times, and excess primary antibody liquid was washed out. The PVDF films were transferred to 10 ml of secondary antibody reaction liquid (goat antimouse IgG (manufactured by Amersham) where 10 µl of peroxidase was added for ATPA detection and goat antimouse IgG (manufactured by Bio-rad) where 10 µl of alkali phosphatase was added for ORF125 detection into 10 ml of blocking solution (M. Iwabuchi et al. Plant Molecular Biology (1999) 39:183-188)), and they were shaken for 1 hour, respectively. The PVDF films were transferred to 100 ml of TTBS (20 mM Tris-HCl (pH 7.5), 500 mM NaCl and 0.05 % Tween20), and they were shaken for 10 minutes. This operation was repeated three times, and an excess secondary antibody was washed out. A chemical emission system "ECL+" (manufactured by Amersham) to peroxidase was used for ATPA detection, and exposure detection was conducted for 5 seconds. BCIP/NBT (manufactured by MOSS Inc.), which is a chromogenic substrate of alkali phosphotase, was used for ORF125 detection, and color development was detected for 5 minutes.

[0162]

As a result, the ATPA accumulation, which is a control, was hardly changed in buds of the diphyletic cytoplasmic male sterility rapeseed, [buds of] fertility restored rapeseed and buds of transformed rapeseed where the DNA was inserted into the cytoplasmic male sterile line; however, the accumulation of ORF125 protein was decreased in the transformed rapeseed dominantly. The degree of this decrease is the same level as the fertility restored line where the fertility restoring gene was introduced due to the cytoplasmic male sterile line by breeding (Fig. 4 and M. Iwabuchi et al. Plant Molecular Biology (1999) 39:183-188). Further, comparing the degree of decrease in ORF125 protein accumulation with the dilute line, it was ascertained that the decrease was 1/8 to 1/16 with the fertility restored rapeseed and approximately 1/8 with transformed rapeseed. As described above, since the fertility restoration in rapeseed and the decrease in ORF125 protein accumulation were strongly linked and in the agreement relationship, it was proved that the DNA sequence functions to decrease the ORF125 protein accumulation within mitochondria, and is a genome DNA sequence where the fertility restoring genes are retained.

In addition, anthers were extracted from a bloomed body and they were observed with a microscope, and it was confirmed that normal pollen was formed (Fig. 5).

[0163]

Embodiment 9: cDNA isolation

A F_2 individuals, which have the Rf1 gene in homo and has pollen fertility, were selected from the F_2 aggregation, which was used at the time of production of gene mal, as RNA donor, and after mRNA was purified from buds, cDNA was synthesized, and cDNA was isolated using 5'-RACE or 3'-RACE method.

(Purification of mRNA)

All RNAs were extracted from buds of F_2 individual, which has the Rf1 gene having allele and has pollen fertility, by a guanidium thiothiocyanate method, which is a usual method, using the RNeasy kit (by Qiagen). PolyA + RNA were purified from all RNAs using mRNA Purification kit (by Amersham Pharmacia) where origo (dT) cellulose columns, and mRNA was obtained.

[0164]

(Isolation of cDNA by 5'-RACE and 3'-RACE)

Using 1 μg of purified mRNA, cDNA was isolated using "Marathon RACE system 5'RACE 3'RACE" kit by 5'-RACE and 3'-RACE methods. As gene specific primers, 5'-GATTCCTTTCTCTTGCATTTCAG-3' (SEQ ID NO 10) and 5'-ATCTCGTCCTTTACCTTCTGTGG-3' (SEQ ID NO 11) were used for 5'-RACE and 3'-RACE, respectively. After base sequences of the obtained clone were defined, cDNA sequences were obtained (SEQ ID NO 2).

[0165]

Embodiment 10: Conversion of cDNA to amino acid sequence and analysis (1) Using a normal genetic code, cDNA was converted into the amino acid sequences using gene analysis software "Genetyx-SV" (Software Development Co., Ltd.), and amino acid sequences described in SEQ ID NO 3 were obtained. PPR motif was analyzed using a program in Protein families' database of alignments and HMNs (hereafter, abbreviated as Pfam: http//:www.sanger.ac.uk/Software/Pfam/search.shtml). As a result of analysis, it was discovered that a translated product of fertility restoring genes shown with SEQ ID NO 1 was a protein having 16 PPR motifs. Further, the PPR motifs were three PPR clusters. The three clusters were as follows:

① PPR cluster #1: PPR cluster where the 1st PPR motif from the N-terminal to the 5th PPR motif comprises successive 175 residues;

② PPR cluster #2: PPR cluster where the 6th PPR motif from the N-terminal to the 12th PPR motif comprises successive 245 residues;
③ PPR cluster #3: PPR cluster where the 13th PPR motif from the N-terminal to the 16th PPR motif comprises successive 140 residues;

[0166]

Embodiment 11: Analysis of protein of the present invention

An experiment about whether or not translation inhibition occurred in Escherichia coli combining a transcriptional product (mRNA) of ORF125, which is a causal protein occurring Kosena cytoplasmic male sterility was conducted.

The fertility restoring genes shown in SEQ ID NO 2 were introduced into a BamHI-SphI section of an Escherichia coli expression vector pQE-80L (by Qiagen), and six histidine residues structured an expression vector to be added to (6XHis) N-terminal (pQEB1/cds6). Further, DNA was amplified using a primer to introduce BamHI section: CGGGATCCGCTCACAATT (SEQ ID NO 12) and an M13 primer RV (manufactured by Takara Shuzo), using pSTV29 (manufactured by Takara Shuzo) vector as a mold. For the DNA amplification, Takara LA PCR Kit (manufactured by Takara Shuzo) was used. After the amplified DNA was cut by restriction enzymes BamHI and EcoRI (manufactured by Takara Shuzo), they were purified using Suprec-02 (manufactured by Takara Shuzo). In order to synthesize DNA fragments having BamHI and EcoRI sections in the 5'-UTR region of ORF125 gene and at the both sides of 25 amino acids of ORF125, PCR was conducted using two primers in accordance with Fujimoto method (PCR experimental protocol of plants: Actuality of synthesized DNA, pp. 84-87 (Shujunsha). For the primers,

125-5'BamHI:

GCGGATCCCAATTTCATTCTGCATCACTCTCCCTGTCGTTATCGACCTCG

CAAGGTTTTTGAAACGGCCGAAACGGGAAGTGACAATACCGCTTTTCTT
C (SEQ ID NO 13) and
125-5'EcoRI:
GGAATTCACTAACTTTACATTCAGTAGGAGTGAGATTATGACAAAAAGTG
GACAATTTTTCGAAAAAGGTAATCATGCATTTATATGCTGAAGAAAAGCG
(SEQ ID NO 14) were used. The amplified DNA was cut by the restriction enzymes BamHI and EcoRI (manufactured by Takara Shuzo), DNA was purified using Suprec-02 (manufactured by Takara Shuzo). The purified DNA was joined using TaKaRa Ligation kit (manufactured by Takara Shuzo), and was transformed to Escherichia coli DH10B (manufactured by Gibco BRL). They were cultured in the LB agar culture medium (1 % Bacto-Tryptone, 0.5 % Bacto-Yeast Extract, 1 % NaCl, 1.5 % Bacto-Agar, 0.1 mM IPTG and 20 ug/ml X-Gal) where 50 μg/ml of chloramphenicol (manufactured by Sigma) was added, at 37 degrees for 18 hours or longer. Plasmid was extracted from a thin blue colony using a usual method, and base sequences were confirmed. As described above, a vector where the 5'-UTR region of ORF125 gene and 174bp including 25 amino acid of ORF125 (the 7th to 180th base sequences output the base sequences described in Fig. 6) were introduced between from the EcoRI section to the transcription starting point of lacZ gene was structured (pSTV125-5' #LA6). Further, simultaneously, a vector having fragments (the 7th to 183rd of the base sequences described in Fig. 6) causing the mutation to a portion equivalent to said 174bp was obtained (pSTV125-5' #LA12).

[0167]

Said vectors pSTV125-5' #LA6 and #LA12 were introduced into the Escherichia coli (manufactured by GibcoBRL), respectively, and they were stationarily cultured in the agar culture medium where 50 μg/ml chloramphenicol (manufactured by Wako Pure Chemicals Industries), 200 μM IPTG (manufactured by Wako Pure Chemicals Industries) and 40 μg/ml

X-Gal (manufactured by Takara Shuzo) were added to the LB culture medium at 37 degrees over night, and a colony was grown, and it became thin blue. In other words, even in the Escherichia coli where any vector was introduced, it was the introduced LacZ genes that were expressed.

[0168]

In addition, in order to introduce the pSTV125-5' vector and the pQEB1/cds6 vector into the Escherichia coli as similar to the above-mentioned, they were cultured using a culture medium where 50 μg/ml ampicillin was added to the above-mentioned culture medium. When pSTV125-5' #LA6 was co-existed, the colony became white; however, when pSTV125-5' #LA12 having mutation to the introduced fragments was co-existed, the colony became thin blue, and the degree of blue was the same as the case of #LA12 singularity. Furthermore, whether or not these introduced vectors fell off from the Escherichia coli was confirmed by extracting the vectors using a usual method after each colony was cultured.

[0169]

According to the above-mentioned results, it seems that the protein expressed within the Escherichia coli by the pQEB1/cds6 vectors became white colony, because the expression of LacZ genes was restrained as a result of combining with mRNA of pSTV125-5' #LA6. Further, when pSTV125-5' #LA12 co-existed, pQEB1/cds6-derived protein could not be combined with mRNA because the introduced fragment section had mutation, and as a result, the LacZ gene was expressed and the color became blue.

[0170]

As described above, it is assumed that the protein having the amino acid sequences described in SEQ ID NO 3 participates in mRNA of ORF125; to be more specific, the transcriptional products of at least ORF125-5'UTR

region and the code region of 25 amino acid residues of ORF125, and the expression of ORF125 protein appears to be restrained.

[0171]

In other words, it is assumed that since the translational products of genes of the present invention are combined with male-sterile genes and inhibit the translation within mitochondria after being transferred to the mitochondria, the accumulation of causal protein of cytoplasmic male sterility is decreased, and the cytoplasmic male sterility is restored to the fertility.

[0172]

[Efficacy of the Invention]

According to the present invention, the Rf genes, particularly, radish-derived Rf1 gene was isolated, and its structure was identified. In addition, according to the present invention, it has become possible to provide a means to establish a rapeseed restoration line by utilizing the isolated Rf genes.

[0173]

[Sequence Listing]

SEQUENCE LISTING

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[0174]

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ttg atg gct acc aag ggc tgc tct ccc aac cta atc act ttc aat act 1200
Leu Met Ala Thr Lys Gly Cys Ser Pro Asn Leu Ile Thr Phe Asn Thr
385                 390                 395                 400
ctc ata gac gga tat tgt ggg gct aag agg ata gat gat gga atg gaa
```

```
Leu Ile Asp Gly Tyr Cys Gly Ala Lys Arg Ile Asp Asp Gly Met Glu
                405                 410                 415
ctt ctc cat gag atg act gaa aca gga tta gtt gct gac aca act act
Leu Leu His Glu Met Thr Glu Thr Gly Leu Val Ala Asp Thr Thr Thr
            420                 425                 430
tac aac act ctt att cac ggg ttc tat ctg gtg ggc gat ctt aat gct
Tyr Asn Thr Leu Ile His Gly Phe Tyr Leu Val Gly Asp Leu Asn Ala
        435                 440                 445
gct cta gac ctt tta caa gag atg atc tct agt ggt ttg tgc cct gat
Ala Leu Asp Leu Leu Gln Glu Met Ile Ser Ser Gly Leu Cys Pro Asp
    450                 455                 460
atc gtt act tgt gac act ttg ctg gat ggt ctc tgc gat aat ggg aaa 1440
Ile Val Thr Cys Asp Thr Leu Leu Asp Gly Leu Cys Asp Asn Gly Lys
465                 470                 475                 480
cta aaa gat gca ttg gaa atg ttt aag gtt atg cag aag agt aag aag
Leu Lys Asp Ala Leu Glu Met Phe Lys Val Met Gln Lys Ser Lys Lys
                485                 490                 495
gat ctt gat gct agt cac ccc ttc aat ggt gtg gaa cct gat gtt caa
Asp Leu Asp Ala Ser His Pro Phe Asn Gly Val Glu Pro Asp Val Gln
            500                 505                 510
act tac aat ata ttg atc agc ggc ttg atc aat gaa ggg aag ttt tta
Thr Tyr Asn Ile Leu Ile Ser Gly Leu Ile Asn Glu Gly Lys Phe Leu
        515                 520                 525
gag gcc gag gaa tta tac gag gag atg ccc cac agg ggt ata gtc cca
Glu Ala Glu Glu Leu Tyr Glu Glu Met Pro His Arg Gly Ile Val Pro
    530                 535                 540
gat act atc acc tat agc tca atg atc gat gga tta tgc aag cag agc 1680
Asp Thr Ile Thr Tyr Ser Ser Met Ile Asp Gly Leu Cys Lys Gln Ser
545                 550                 555                 560
```

```
cgc cta gat gag gct aca caa atg ttt gat tcg atg ggt agc aag agc
Arg Leu Asp Glu Ala Thr Gln Met Phe Asp Ser Met Gly Ser Lys Ser
                565                 570                 575
ttc tct cca aac gta gtg acc ttt act aca ctc att aat ggc tac tgt
Phe Ser Pro Asn Val Val Thr Phe Thr Thr Leu Ile Asn Gly Tyr Cys
            580                 585                 590
aag gca gga agg gtt gat gat ggg ctg gag ctt ttc tgc gag atg ggt
Lys Ala Gly Arg Val Asp Asp Gly Leu Glu Leu Phe Cys Glu Met Gly
        595                 600                 605
cga aga ggg ata gtt gct aac gca att act tac atc act ttg att tgt
Arg Arg Gly Ile Val Ala Asn Ala Ile Thr Tyr Ile Thr Leu Ile Cys
    610                 615                 620
ggt ttt cgt aaa gtg ggt aat att aat ggg gct cta gac att ttc cag  1920
Gly Phe Arg Lys Val Gly Asn Ile Asn Gly Ala Leu Asp Ile Phe Gln
625                 630                 635                 640
gag atg att tca agt ggt gtg tat cct gat acc att acc atc cgc aat  1968
Glu Met Ile Ser Ser Gly Val Tyr Pro Asp Thr Ile Thr Ile Arg Asn
                645                 650                 655
atg ctg act ggt tta tgg agt aaa gag gaa cta aaa agg gca gtg gca  2016
Met Leu Thr Gly Leu Trp Ser Lys Glu Glu Leu Lys Arg Ala Val Ala
            660                 665                 670
atg ctt gag aaa ctg cag atg agt atg gat cta tca ttt ggg gga tga  2064
Met Leu Glu Lys Leu Gln Met Ser Met Asp Leu Ser Phe Gly Gly
        675                 680                 685
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[0176]

<210> 3

<211> 687

<212> PRT

<213> Raphanus sativus

<400> 3

Met Leu Ala Arg Val Cys Gly Phe Lys Cys Ser Ser Ser Pro Ala Glu
1 5 10 15

Ser Ala Ala Arg Leu Phe Cys Thr Arg Ser Ile Arg Asp Thr Leu Ala
20 25 30

Lys Ala Ser Gly Glu Ser Cys Glu Ala Gly Phe Gly Gly Glu Ser Leu
35 40 45

Lys Leu Gln Ser Gly Phe His Glu Ile Lys Gly Leu Glu Asp Ala Ile
50 55 60

Asp Leu Phe Ser Asp Met Leu Arg Ser Arg Pro Leu Pro Ser Val Val
65 70 75 80

Asp Phe Cys Lys Leu Met Gly Val Val Val Arg Met Glu Arg Pro Asp
85 90 95

Leu Val Ile Ser Leu Tyr Gln Lys Met Glu Arg Lys Gln Ile Arg Cys
100 105 110

Asp Ile Tyr Ser Phe Asn Ile Leu Ile Lys Cys Phe Cys Ser Cys Ser
115 120 125

Lys Leu Pro Phe Ala Leu Ser Thr Phe Gly Lys Ile Thr Lys Leu Gly
130 135 140

Leu His Pro Asp Val Val Thr Phe Thr Thr Leu Leu His Gly Leu Cys
145 150 155 160

Val Glu Asp Arg Val Ser Glu Ala Leu Asp Phe Phe His Gln Met Phe
165 170 175

Glu Thr Thr Cys Arg Pro Asn Val Val Thr Phe Thr Thr Leu Met Asn
180 185 190

Gly Leu Cys Arg Glu Gly Arg Ile Val Glu Ala Val Ala Leu Leu Asp
195 200 205

Arg Met Met Glu Asp Gly Leu Gln Pr Thr Gln Ile Thr Tyr Gly Thr
210 215 220

Ile Val Asp Gly Met Cys Lys Lys Gly Asp Thr Val Ser Ala Leu Asn
225 230 235 240
Leu Leu Arg Lys Met Glu Glu Val Ser His Ile Ile Pr Asn Val Val
245 250 255
Ile Tyr Ser Ala Ile Ile Asp Ser Leu Cys Lys Asp Gly Arg His Ser
260 265 270
Asp Ala Gln Asn Leu Phe Thr Glu Met Gln Glu Lys Gly Ile Phe Pro
275 280 285
Asp Leu Phe Thr Tyr Asn Ser Met Ile Val Gly Phe Cys Ser Ser Gly
290 295 300
Arg Trp Ser Asp Ala Glu Gln Leu Leu Gln Glu Met Leu Glu Arg Lys
305 310 315 320
Ile Ser Pro Asp Val Val Thr Tyr Asn Ala Leu Ile Asn Ala Phe Val
325 330 335
Lys Glu Gly Lys Phe Phe Glu Ala Glu Glu Leu Tyr Asp Glu Met Leu
340 345 350
Pro Arg Gly Ile Ile Pro Asn Thr Ile Thr Tyr Ser Ser Met Ile Asp
355 360 365
Gly Phe Cys Lys Gln Asn Arg Leu Asp Ala Ala Glu His Met Phe Tyr
370 375 380
Leu Met Ala Thr Lys Gly Cys Ser Pro Asn Leu Ile Thr Phe Asn Thr
385 390 395 400
Leu Ile Asp Gly Tyr Cys Gly Ala Lys Arg Ile Asp Asp Gly Met Glu
405 410 415
Leu Leu His Glu Met Thr Glu Thr Gly Leu Val Ala Asp Thr Thr Thr
420 425 430
Tyr Asn Thr Leu Ile His Gly Phe Tyr Leu Val Gly Asp Leu Asn Ala
435 440 445
Ala Leu Asp Leu Leu Gln Glu Met Ile Ser Ser Gly Leu Cys Pro Asp

```
    450                 455                 460
Ile Val Thr Cys Asp Thr Leu Leu Asp Gly Leu Cys Asp Asn Gly Lys
465                 470                 475                 480
Leu Lys Asp Ala Leu Glu Met Phe Lys Val Met Gln Lys Ser Lys Lys
                485                 490                 495
Asp Leu Asp Ala Ser His Pro Phe Asn Gly Val Glu Pro Asp Val Gln
            500                 505                 510
Thr Tyr Asn Ile Leu Ile Ser Gly Leu Ile Asn Glu Gly Lys Phe Leu
        515                 520                 525
Glu Ala Glu Glu Leu Tyr Glu Glu Met Pro His Arg Gly Ile Val Pro
    530                 535                 540
Asp Thr Ile Thr Tyr Ser Ser Met Ile Asp Gly Leu Cys Lys Gln Ser
545                 550                 555                 560
Arg Leu Asp Glu Ala Thr Gln Met Phe Asp Ser Met Gly Ser Lys Ser
                565                 570                 575
Phe Ser Pro Asn Val Val Thr Phe Thr Thr Leu Ile Asn Gly Tyr Cys
            580                 585                 590
Lys Ala Gly Arg Val Asp Asp Gly Leu Glu Leu Phe Cys Glu Met Gly
        595                 600                 605
Arg Arg Gly Ile Val Ala Asn Ala Ile Thr Tyr Ile Thr Leu Ile Cys
    610                 615                 620
Gly Phe Arg Lys Val Gly Asn Ile Asn Gly Ala Leu Asp Ile Phe Gln
625                 630                 635                 640
Glu Met Ile Ser Ser Gly Val Tyr Pro Asp Thr Ile Thr Ile Arg Asn
                645                 650                 655
Met Leu Thr Gly Leu Trp Ser Lys Glu Glu Leu Lys Arg Ala Val Ala
            660                 665                 670
Met Leu Glu Lys Leu Gln Met Ser Met Asp Leu Ser Phe Gly Gly
        675                 680                 685
```

[0177]

<210> 4

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 4

gaagcaaaaa agaaaacgag cagag 25

[0178]

<210> 5

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 5

ccaaaaatcc gaaatccgaa tagac 25

[0179]

<210> 6

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 6

ctcggctctg ggtttagtga 20

[0180]

<210> 7

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 7

tccacaaacc ctagccaaca 20

[0181]

<210> 8

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 8

gcttatgctt ctctggttcg cctc 24

[0182]

<210> 9

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 9

ctcagttttc gtcaccttac acaatgc 27

[0183]

<210> 10

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 10

gattcctttc tcttgcattt cag 23

[0184]

<210> 11

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 11

atctcgtcct ttaccttctg tgg 23

[0185]

<210> 12

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 12

cgggatccgc tcacaatt 18

[0186]

<210> 13

<211> 100

<212> DNA

<213> Artificial Sequence

<220>

<223> Description f Artificial Sequence: Synthetic DNA

<400> 13

gcggatccca atttcattct gcatcactct ccctgtcgtt atcgacctcg caaggttttt 60

gaaacggccg aaacgggaag tgacaatacc gcttttcttc 100

[0187]

<210> 14

<211> 100

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Synthetic DNA

<400> 14

ggaattcact aactttacat tcagtaggag tgagattatg acaaaaagtg gacaattttt 60

cgaaaaaggt aatcatgcat ttatatgctg aagaaaagcg 100

[Brief Description of Drawings]

Fig. 1 shows an Rf marker gene map.

Fig. 2 shows a pattern diagram of lambda clone CHI structure where base sequences described in SEQ ID NO 1 are sufficiently maintained.

Fig. 3 shows detection results of introduced DNA in the transformant using the PCR method.

Fig. 4 shows analysis results of accumulation decrease in ORF125, which is a cms protein in the transformant.

Fig. 5 shows results where anthers were extracted from flower bodies of transformed rapeseed and they were observed by a microscope.

Fig. 6 shows base sequence of pSTV125-5' #LA6 and pSTV125-5' #LA12.

FIG. 1

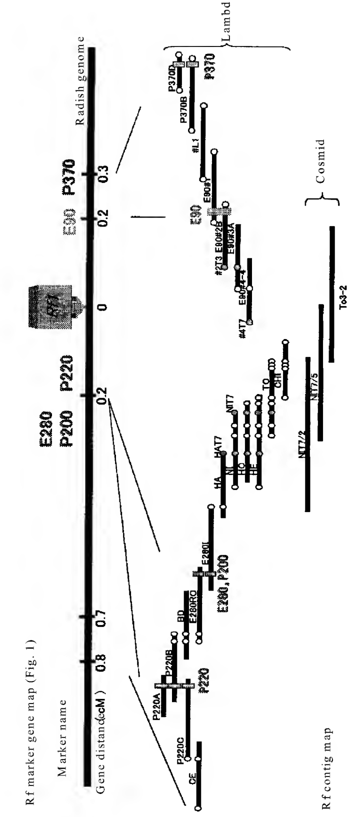

Rf marker gene map (Fig. 1)
Marker name
Gene distance (cM)
Radish genome
P370
E90
P220
E280
P200
0.3
0.2
0
0.2
0.7
0.8
Lambd
Cosmid
Rf contig map
P370D
P370B
P370
#L1
E90#1
E90
E90#2B
E90#3A
#2T3
#4T7
Tc3-2
NIT7/5
NIT7/2
NIT7
HAT7
HA
NI
HO
HE
TO
CHI
E280I
E280, P200
E280RC
BD
P220B
P220A
P220
P220C
CE

FIG.

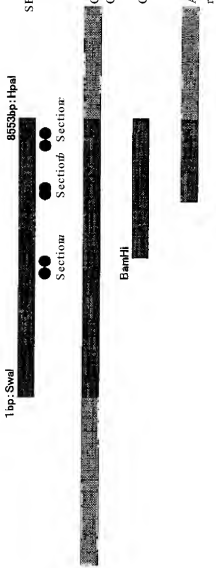
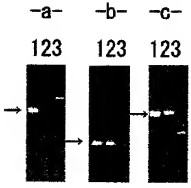

1bp: SwaI
8553bp: HpaI
SEQ ID NO: 8553bp
Section
Section
Section
CHI lambda clone fragments: approximate
Cloning fragments of CHI/pBIGRZ2
BamHI
Cloning fragments of cds6BT/pKM424
A portion assumed as inserted into transfo
rapeseed

FIG. 3



lane 1 Control vector

lane 2 Transformed rapeseed

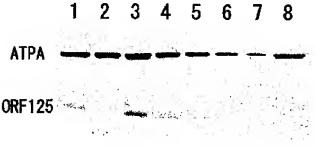
lane 3 Cytoplasmic male sterile rapeseed

a: 3186bp-3753bp length:568bp

b: 4869bp-5112bp length:244bp

c: 7766bp-8250bp length:485bp

FIG. 4



Lane 1: Cytoplasmic male sterile rapeseed -1- 15 μg

Lane 2: Fertility restored rapeseed 15 μg

Lane 3: Cytoplasmic male sterile rapeseed -2- 15 μg

Lanes 4 to 7: Cytoplasmic male sterile rapeseed -2- 15/2 μg, 15/4 μg, 15/8 μg and 15/16 μg of dilute line

Lane 8: Transformed rapeseed 15 μg

FIG. 5

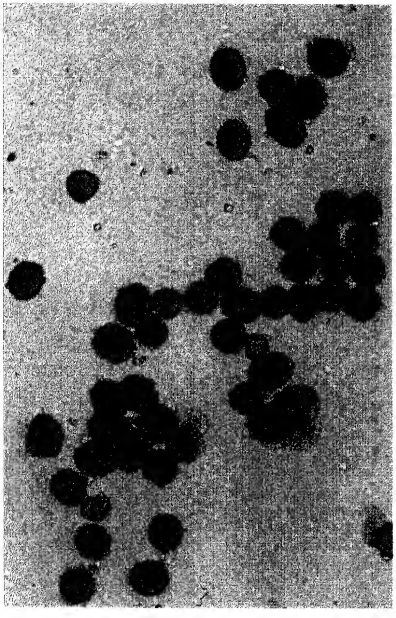


FIG. 6

```
pSTV125-5' #LA12.nuc    1:GGATCCCAATTTCATTCTGCATCACTCTCCCTGTCGTTATGCGACCTCGCAAGGTTTTTG 60
pSTV125-5' #LA6.nuc     1:GGATCCCAATTTCATTCTGCATCACTCTCCCTGTCGTTATCGACCTCG-CAAGGTTTTTG 59
                          ******************************************  *  **********

pSTV125-5' #LA12.nuc   61:AAACGGCCGAAACGGGAAGTGACAATACCGCTTTTCTTGAGCATATAAATCATGATTAC- 119
pSTV125-5' #LA6.nuc    60:AAACGGCCGAAACGGGAAGTGACAATACCGCTTTTCTTCAGCATATAAATGCATGATTAC 119
                          ************************************** **********  *

pSTV125-5' #LA12.nuc  120:CTTTTTTCGAAAAATTGTCCACTTTTTGTCATAATCCTCACTTCCTACTGAATGTAAAGT 179
pSTV125-5' #LA6.nuc   120:CTTTT-TCGAAAAATTGTCCACTTTTTGTCATAATCTCACTCCTACTGAAATTAA-A-GT 176
                          ***** ********************************  * *  * **

pSTV125-5' #LA12.nuc  180:TAGTGAATTC                                              189
pSTV125-5' #LA6.nuc   177:TAGTGAATTC                                              186
                          **********
```

(57) [Abstract]

[Subject]

To isolate Rf genes, particularly radish-derived Rf1 gene, and to identify a structure thereof.

[Resolution means]

Proteins participating in restoration from cytoplasmic male sterility to fertility, wherein the proteins contain 14 or more of pentatricopeptide repeat (hereafter, abbreviated as PPR) motifs; the PPR motif group is divided into three or more blocks; each block has at least two or more PPR motifs, respectively; and the block at a carboxy terminal (C-terminal) has four PPR motifs.

Applicant History Information

Identification Number [000005968]

1. Modified Date October 20, 1994
[Reason for modification] Title change

Address: 5-2, Marunouchi 2-chome, Chiyoda-ku, Tokyo
Name: Mitsubishi Chemical Corporation